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Trabalho efetuado sob a orientação da
Doutora Cândida Lucas
e da
Doutora Célia Ferreira

maio de 2017

DECLARAÇÃO DE INTEGRIDADE

Declaro ter atuado com integridade na elaboração da presente tese. Confirmo que em todo o trabalho conducente à sua elaboração não recorri à prática de plágio ou a qualquer forma de falsificação de resultados.

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Abstract

Saccharomyces cerevisiae Gup1 is a membrane bound *O*-acyltransferase firstly associated with glycerol uptake, and then involved in a wide range of cellular processes, including: (i) plasma membrane and cell wall composition, (ii) rafts assembly and integrity, (iii) lipid metabolism and GPI anchor remodeling, (iv) trafficking, (v) cytoskeleton polarization and budding pattern, (vi) telomere length, (vii) cell death, and (viii) ECM composition among others. *Candida albicans* Gup1 was also associated with morphogenesis and differentiation. The disruption of *GUP1* in this pathogenic yeast reduces virulence, affecting its capacity to adhere/invade, to differentiate into hyphae and to form biofilms. Yeast Gup1 and Gup2 proteins in higher Eukaryotes, respectively HHATL and HHAT, are regulators of the morphogenic cell-cell signalling Hedgehog pathway. HHAT is responsible for the palmitoylation of the Hedgehog secreted morphogen, and HHATL for its negative regulation. The existence of a paracrine signaling pathway similar to Hedgehog was never described in microbial cells. However, unicellular organism can form large aggregates of cells like colonies or biofilms that have a tissue-like behavior, where cells differentiate, specialize, and spatially organize, supported by a complex saccharide and proteinatious ECM. Therefore, cell-cell communication must underlie these numerous communities. It remains unclear, however, whether this occurs through a diffusible chemical, like ammonia or *quorum-sensing* chemicals, or through a peptide signal like the Hh morphogen from higher Eukaryotes. The presence of a Gup/HHAT(L) protein in all Eukaryotes suggests a conserved mechanism in which these proteins might be involved.

The main goal of this work was to identify and characterize the proteins interacting physically with Gup1 in *S. cerevisiae,* as a first step to disclose the function(s) of Gup proteins in yeast. Several proteins were previously suggested to putatively interact with Gup1, though only one did not arise from HTP surveys, the ammonium transceptor Mep2. In this work, two novel Gup1 physical interactions were found: the yeast outer mitochondrial membrane VDAC (Por1), and the eisosome core component Pil1. The interaction between Gup1 and the newly identified Por1 and Pil1 partners, as well as the previously identified Mep2, was studied: (i) the expression and localization of these partners was assessed by RT-PCR and GFP fluorescence respectively, and (ii) several processes commonly associated to Gup1 were evaluated phenotypically, for which purpose new single and double deleted strains were built.

Although the expression of neither Gup1 partner seems to be significantly altered by the deletion of *GUP1*, its absence affects the distribution of Por1, and Mep2. Importantly, the interaction between Gup1 and Por1, proved to be determinant for the nature of acetic acid-induced cell death, which changes from a necrosis-like program in Δ*gup1* cells, to what seems to be an apoptotic-like cell death in the absence of both proteins. In spite of the mitochondrial localization of Por1, its interaction with Gup1 is also important for the control of cell wall integrity, possible through the regulation CWI signaling, and for the differentiation of structured colonies and development of multicellular aggregates/mats. On the other hand, the interaction between Gup1 and the physical partner Pil1 seems to be important for the organization and/or stability of the plasma membrane. In the absence of Gup1, the number of eisosomes was reduced, suggesting an inefficient Pil1 assembly at the membrane, possibly related to the altered levels of phosphoinositide. Moreover, the absence of Pil1 increases the susceptibility of yeast cells to SDS, a phenotype that is exacerbated in Δ*gup1*Δ*pil1* mutants. Finally, Gup1 and Mep2 seem to collaborate in the definition of cell wall composition/structure. Deletion of Mep2 in Δ*gup1* cells increases their sensitivity to some cell wall related stresses, suggesting that Mep2-associated transported and/or signaling could be important for cell survival when the cell wall is affected. Accordingly, both proteins appear to be essential to adherence/invasive growth of yeast cells.

The work developed in this thesis represents the first systematic effort to identify Gup1 physical interactors, and a first step to understand the biological relevance and the niche of these interactions. Previous data suggest that yeast Gup1 is, or locates at, a hub between CWI, TORC1, TORC2/YPK, and HOG pathways. The present work results are compatible with this possibility, and highlight the intricate and complex role of Gup proteins in yeast cells, by showing that Gup1 interacts with mitochondrial, membrane and eisosomal proteins in the regulation of processes as different as cell death or plasma membrane and cell wall composition/organization.

Resumo

A proteína Gup1 de *Saccharomyces cerevisiae* é uma *O*-aciltransferase membranar, inicialmente associada ao transporte de glicerol e, mais tarde, a uma grande variedade de processos celulares, incluindo: (i) composição da membrana plasmática e parede celular, (ii) montagem e estabilidade de *rafts*, (iii) metabolismo de lípidos e remodelação de caudas GPI, (iv) tráfego intracelular, (v) polarização do citoesqueleto e padrão de gemulação, (vi) comprimento de telómeros, (vii) morte celular, e (viii) composição da matriz extracelular, entre outros. Em *Candida albicans*, a proteina Gup1 foi também associada à morfogénese e diferenciação. A deleção do *GUP1* reduz a virulência desta levedura patogénica, afectando a sua capacidade para aderir/invadir, para se diferenciar em hifas e para formar biofilmes. Em Eucariotas superiores, as proteínas HHATL e HHAT, homólogos do Gup1 e do Gup2 de *S. cerevisiae*, respectivamente, são reguladores da via de sinalização Hedgehog. O HHAT é responsável pela palmitoilação do morfogéneo Hedgehog (Hh), enquanto o HHATL funciona como regulador negativo. A existência de uma via de sinalização semelhante à Hedgehog nunca foi descrita em células microbianas. Ainda assim, organismos unicelulares podem formar grandes agregados celulares, como colónias ou biofilmes, apresentando um comportamento semelhante ao de um tecido, nos quais as células se diferenciam, especializam e organizam espacialmente, suportadas por uma complexa matrix extracelular de natureza sacarídea e proteica. A comunicação intercelular deverá ser fundamental nestas comunidades. No entanto, é ainda desconhecido se esta comunicação ocorre através da difusão de compostos químicos, como a amónia ou *quorum-sensing*, ou através de um sinal peptídico, como o morfogéneo Hh de Eucariotas superiores. A existência de uma proteína Gup/HHAT(L) em todos os Eucariotas, sugere um mecanismo conservado no qual estas proteínas estarão envolvidas.

Este trabalho teve como principal objectivo a identificação e caracterização de proteínas que interagem fisicamente com o Gup1 em *S. cerevisiae*, como primeiro passo para desvendar as funções do Gup1 em leveduras. Várias proteínas foram anteriormente identificadas como prováveis parceiros do Gup1, embora todas excepto uma, o transportador de amónio Mep2, tenham sido identificadas em ensaios HTP. Neste trabalho, foram identificadas duas novas interacções físicas com o Gup1: a proteína VDAC da membrana mitocondrial externa – Por1, e um componente dos eisossomas –

Pil1. As interacções entre o Gup1 e estes parceiros, Por1 e Pil1, bem como a interacção com a Mep2, foram estudadas a nível de: (i) expressão e localização dos parceiros, avaliadas por RT-PCR e fluorescência de GFP, respectivamente; e (ii) de vários processos celulares associados ao Gup1, através de avaliação fenotípica.

Apesar da expressão dos vários parceiros do Gup1 não ser significativamente alterada pela deleção do *GUP1*, a ausência desta proteína afecta a distribuição da Por1 e da Mep2. A interacção entre Gup1 e Por1 parece ainda ser determinante para definir a natureza da morte celular induzida por ácido acético, a qual altera de um processo do tipo necrótico em células *Δgup1*, para o que parece ser um processo do tipo apoptótico em células *Δgup1Δpor1*. Apesar da localização mitocondrial da Por1, a sua interacção com o Gup1 é ainda importante para o controlo da integridade da parede celular, possivelmente através da regulação da via de sinalização CWI, e para a diferenciação de colónias estruturadas e desenvolvimento de agregados multicelulares. Por outro lado, a interacção entre Gup1 e o parceiro Pil1 parece ser importante para a organização e/ou estabilidade da membrana plasmática. Na ausência do Gup1, o número de eisossomas é reduzido, sugerindo defeitos na associação da Pil1 à membrana. Para além disso, a ausência da Pil1 aumenta a susceptibilidade das células de levedura ao SDS, um fenótipo que é agravado no mutante *Δgup1Δpil1*. Finalmente, as proteínas Gup1 e Mep2 parecem colaborar na estabilidade da parede celular. A deleção do *MEP2* em células *Δgup1* aumenta a sensibilidade a alguns stresses associados à parede, o que sugere que o transporte e/ou sinalização através da Mep2 podem ser importantes para a sobrevivência celular quando a parede está afectada. Ambas as proteínas parecem ser ainda essenciais para a aderência/crescimento invasivo das células de levedura.

O trabalho desenvolvido nesta tese consiste no primeiro esforço sistemático para identificar parceiros físicos do Gup1 no sentido de compreender a relevância biológica destas interacções. Trabalhos anteriores ”colocam” o Gup1 no cruzamento entre as vias CWI, TORC1, TORC2/YPK e HOG. Os resultados deste estudo são compatíveis com esta possibilidade, e destacam o papel complexo das proteínas Gup em leveduras, demonstrando a interacção do Gup1 com proteínas mitocondriais, da membrana plasmática e eisossomais na regulação de processos tão distintos quanto a morte celular ou a composição/organização da membrana ou parece celular.

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Abbreviations List

CFW	calcofluor white
CLS	chronological life span
CR	congo red
CWI/PKC	cell wall integrity/ protein kinase C
cyt *c*	cytochrome c
Co-IP	Co-immunoprecipitation
c.f.u.	colony forming unit
Da (kDa)	dalton (kilodalton)
DAG	diacylglycerol
DRM	detergent-resistant microdomains
ECM	extracellular matrix
ER	endoplasmic reticulum
GFP	green fluorescence protein
H_2O_2	hydrogen peroxide
Hh	hedgehog
HHAT	hedgehog acyltransferase
HHATL	hedgehog acyltransferase like
HOG	high osmolarity glycerol
LCB	long chain base (lipids)
MAPK	mitogen activated protein kinase
MBOAT	membrane-bound *O*-acyltransferases
MCC	membrane compartments of Can1
MCP	membrane compartments of Pma1
MCT	membrane compartments of TORC2
NaCl	sodium chloride
PCD	programmed cell death
PI	propidium iodide
PI(4,5)P2	phosphoinositol 4,5-biphosphate
ROS	reactive oxygen species
TAG	triacylglycerol
TF	transcription factors
TMD	transmembrane domain

TOR	target of rapamycin
UPR	unfolded protein response
VDAC	voltage-dependent anion channel
Wnt	wingless

CHAPTER 1

General Introduction

This chapter contains parts of the following publication:

Cândida Lucas, Célia Ferreira, Giulia Cazzanelli, Ricardo Franco-Duarte, **Joana Tulha** (2016). Yeast Gup1(2) Proteins Are Homologues of the Hedgehog Morphogens Acyltransferases HHAT(L): Facts and Implications. *J. Dev. Biol.*, *4*(4), 33

Yeast: Life in community

The yeast *Saccharomyces cerevisiae* is a recognized model to enlighten higher eukaryotic molecular processes, including the ones underlying human pathologies, which are very far from microbial life (Verduyckt *et al.*, 2016). Intuitively, we associate yeasts, as microbes, to a planktonic form of life. However, microbes in the wild live mostly as large communities of cells forming biofilms or colonies, the biology of which remains largely unknown (Brückner and Mösch, 2012). A colony or a biofilm displays a *proto-tissue* complex behaviour (Čáp *et al.*, 2012). In these communities, cells organize spatially, morphologically and functionally to ensure the survival of the group. This implies the outlying orchestrated differentiation and death of cells to accomplish an efficient colonization of the substrate, be it the pulp of a fruit or the surface of a medical device.

Within the large multicellular communities of yeast, cells behave similarly to their higher eukaryotic counterparts. They are born, grow larger and age while replicating, until they senesce and die. Alternatively, they may die young, following an apoptotic cell death program (Váchová and Palková, 2005, 2007), allowing the supply of nutrients to the inner layers of the group (Váchová and Palková, 2005), located farther from the nutrient richer environment. Additionally, yeast cells may differentiate, shifting from yeast into true or pseudo-hyphae. These differ morphologically and physiologically (Váchová *et al.*, 2009; St'ovíček *et al.*, 2010; Turrà *et al.*, 2016). The differentiation shift promotes an invasive behaviour generally associated with strain virulence (Zupan and Raspor, 2010). Therefore, in contrast to planktonic growth, the survival strategy is collective (Brückner and Mösch, 2012) and resembles tissues from higher eukaryotes. Accordingly, large populations of yeast cells are imbedded in an extracellular matrix (ECM) composed of polysaccharides (Faria-Oliveira *et al.*, 2015a) and a large proteome (Faria-Oliveira *et al.*, 2015b), including many representatives of higher eukaryote ECM key proteins (Faria-Oliveira *et al.*, 2015b).

In higher eukaryotes, long distance communication between cells is achieved by secreted signalling proteins, namely from the Hedgehog (Hh) or Wingless (Wnt) pathways. These ligands interact with specific membrane-resident receptors that trigger a downstream signalling pathway in the receiving cell. No yeast cell-to-cell molecular

communication vehicle has been recognized so far, although ammonia gradients were suggested to perform a role in the collective orchestration of cell behaviour (Palková and Vachova, 2003). Moreover, the secretion of quorum-sensing small chemicals has been described in yeasts and suggested to perform a role in the control of population density (Chen *et al.*, 2004; Sprague and Winans, 2006). Concomitantly, neither Hh- or Wnt-like pathways were described in yeasts.

The maturation of the Hh secreted morphogens involves intein self-splicing and C- and N-terminal lipidation (reviewed by Guerrero and Kornberg (2014)). In *S. cerevisiae* there is only one recognized self-splicing protein that has a Hh-like Hint domain, the endonuclease VDE (PI-SceI). Vde is a small intein derived from self-splicing of the Vma1 subunit of the vacuolar ATPase, which is required for gene conversion during meiosis (Fukuda *et al.*, 2004). Moreover, the highly homologous yeast proteins Gup1 and Gup2, discovered by our group in 2000 (Holst *et al.*, 2000), have two homologues in higher eukaryotes that are responsible for the Hh-secreted morphogen N-terminal palmitoylation (HHAT – Hedgehog acyltransferase/Gup2) (Chamoun *et al.*, 2001; Buglino and Resh, 2008), and the negative regulation of the pathway (HHATL - Hedgehog acyltransferase like/Gup1) (Abe *et al.*, 2008). Gup1 and Gup2 are members of the MBOAT superfamily of membrane bound *O*-acyltransferases (Hofmann, 2000; Neves *et al.*, 2004b) and are present in all eukaryotic genomes sequenced to date. In animal cells, the nomenclature is not consistent: in mouse, human or fly cells, both Gup1 and Gup2 are known by numerous aliases (Table 1). In yeast, their designation as GUP (Glycerol Uptake Protein) came from their influence on the performance of glycerol active transport (Holst *et al.*, 2000; Neves *et al.*, 2004a), which is actually accomplished by Stl1 (Ferreira *et al.*, 2005).

In yeasts, the Gup proteins were implicated in a vast number of phenotypes from very diverse but fundamental biological/molecular processes. The information available from *S. cerevisiae* and from another model yeast, the human commensal/opportunistic pathogen *Candida albicans*, as well as the role of Gup homologues in the regulation of Hh ligand secretion in higher eukaryotes, will be presented in the next sections.

Table 1- Gup1 and Gup2 aliases in the literature and databases. Table published in Lucas *et al.*, 2016.

Aliases		Organism	Key References
Gup1	Gup2	*Saccharomyces cerevisiae*	(Holst *et al.*, 2000)
Gup1	-	*Candida albicans*	(Ferreira *et al.*, 2010)
-	RASP	*Drosophila melanogaster*	(Micchelli *et al.*, 2002)
	Skinny Hedgehog		(Chamoun *et al.*, 2001)
	Sightless		(Lee *et al.*, 2001)
	Central Missing		(Amanai and Jiang, 2001)
HHATL	HHAT	*Mus musculus*	(Abe *et al.*, 2008)
HHATL	HHAT	*Homo sapiens*	(NCBI)
c3orf3			(Soejima *et al.*, 2001)
KIAA117			(Zhang *et al.*, 2005)
MBOAT3 *			(NCBI)
MSTP002 #			(NCBI)
OACT3 *			(NCBI)

* Nomenclature shared with *Xenopus laevis* and *X. tropicalis.* # Nomenclature shared with *X. tropicalis*
NCBI: https://www.ncbi.nlm.nih.gov/

Yeast Gup1 and Gup2 Proteins

Gup1 and Gup2 Are Members of the MBOAT Superfamily

S. cerevisiae GUP1 was firstly associated with the recovery of glycerol from the medium for osmoregulation purposes (Holst *et al.*, 2000). In accordance, *GUP1*-deleted mutants are deficient in glycerol active transport (Holst *et al.*, 2000; Neves *et al.*, 2004a). Still, the glycerol active permease is encoded by the gene *STL1* (Ferreira and Lucas, 2008). The involvement of Gup1 on glycerol transport was interpreted as an indirect effect resulting from the involvement of Gup1 in lipid raft formation (Ferreira and Lucas, 2008). Raft disassembly causes the misdistribution of Pma1 H^+-ATPase plasma membrane (Bagnat *et al.*, 2001; Ferreira and Lucas, 2008), possibly decreasing the proton motive force required for proper function of active transporters like Stl1 as suggested by Ferreira and Lucas (2008). No further research was performed to confirm that hypothesis.

Gup1 and his close homologue Gup2 share a high degree of similarity (77%) and identity (57%) (Holst *et al.*, 2000). These proteins are members of the MBOAT superfamily of multispanning membrane-bound *O*-acyltransferases, a superfamily that was first suggested based exclusively on sequence similarity between a small group of proteins (Hofmann, 2000). Besides Gup proteins from *S. cerevisiae*, the MBOAT superfamily includes mammalian acyl-CoA:cholesterol acyltransferases (ACAT), fly palmitoyltransferases (Porcn and Nessy), *Arabidopsis thaliana* diglyceride acyltransferases (DGAT), bacteria *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* D-alanyltransferases (DltB), and alginate *O*-acetyltransferases (AlgI) (Hofmann, 2000). All of these proteins share a highly-conserved histidine residue localized in a hydrophobic domain (His^{447} in yeast Gup1), as well as another residue of histidine, asparagine or aspartic acid, localized 30–50 amino acids upstream within a hydrophilic region (His^{411} in the yeast Gup1). These conserved positions should correspond to the active centre of these enzymes (Hofmann, 2000). The MBOAT superfamily is presently subdivided into three functionally different subgroups: (1) one group includes enzymes involved in neutral lipid biosynthesis (ACATs (acyl-coenzyme A:cholesterol acyltransferases) and DGATs (diacylglycerol *O*-acyltransferases)), (2) another group includes proteins involved in phospholipid remodelling (LPATs (lysophosphatidate acyltransferases)), and (3) a third group includes the enzymes implicated in protein/peptide acylation (Porcupine from the Wnt pathway, HHAT(L) from the Hh pathway, and GOAT (Ghrelin *O*-acyltransferase) from the insulin regulatory pathway) (Chang *et al.*, 2011). Gup1, although included in the third group, has also been considered a member of the LPATs group due to the multiple biological and molecular roles that can be assigned to it.

In the mammalian HHATL, the highly conserved His residue indispensable to the acyltransferase activity of the MBOAT superfamily has been replaced with a Leu residue (Fig. 1). Interestingly, in *S. cerevisiae* Gup1, the engineered substitution of His^{447} by a leucine caused loss of phenotype (Bleve *et al.*, 2011), raising the question whether mammalian HHATL actually functions as an acyltransferase (Abe *et al.*, 2008). The roles attributed to Gup1 in all cellular models are for now exclusively biological/phenomenological, not biochemical. In contrast, the HHAT from high eukaryotes has a fully recognized enzymatic function in the N-palmitoylation of the Hh-secreted signals that was reproduced *in vitro* (Buglino and Resh, 2008, 2010).

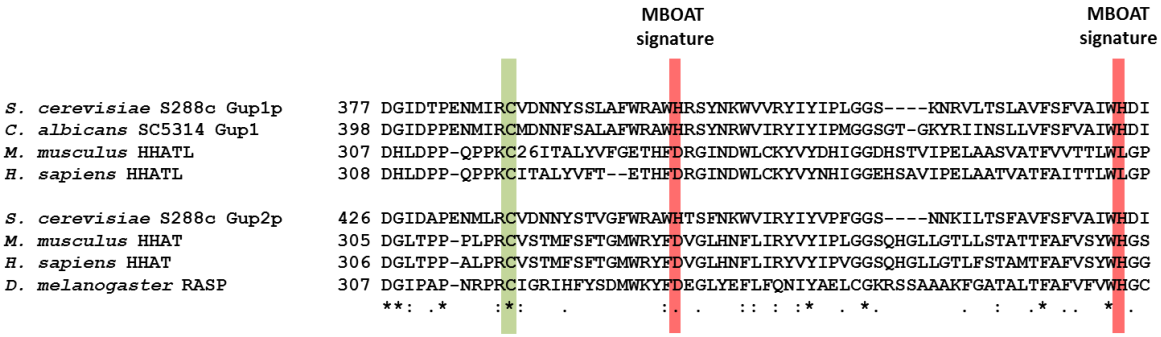

Figure 1 - Alignment of the most highly conserved region of *S. cerevisiae* Gup proteins with its orthologues from *C. albicans*, *M. musculus* as *H. sapiens* and *D. melanogaster*. The putative catalytic asparagine and histidine residues are shaded in red. A conserved cysteine present in all sequences and predicted to be palmitoylated are shaded in green.

MBOAT family proteins share a common topology. They all have 8–12 hydrophobic/transmembrane domains (TMDs) and localize in the ER or Golgi (Chang and Magee, 2009; Chang *et al.*, 2011; Masumoto *et al.*, 2015). According to the most common algorithms used to calculate the hydrophobicity of amino acid sequences, the *S. cerevisiae* Gup1 and Gup2 proteins both have 10 well-defined TMD. This number of TMD implicates that Gup1 terminals are both located on the same side of the membrane. However, the Gup1 C- and N-terminal domains were experimentally determined to localize, respectively, in the periplasmic and cytosolic sides of the membrane (Bleve *et al.*, 2005).

HHAT was first predicted to have 8 TMDs (Buglino and Resh, 2010), but this topology seems inconsistent when compared to other members of the MBOAT family. Specifically, according to the predicted structure for HHAT, the MBOAT signature amino acid residues would be located in the cytoplasm, in which case it would be a feature unique to HHAT among all the other MBOAT members (Konitsiotis *et al.*, 2015). Two independent research groups (Konitsiotis *et al.*, 2015; Matevossian and Resh, 2015) predicted the topology of human HHAT based on updated prediction software, to have 10 TMDs and two re-entrant loops (Fig. 2). The invariant His appears located in the lumenal side of the ER, whereas the Asp residue locates on the opposite side. Conceivably, HHATL should have a similar structure, due to the high sequence similarity.

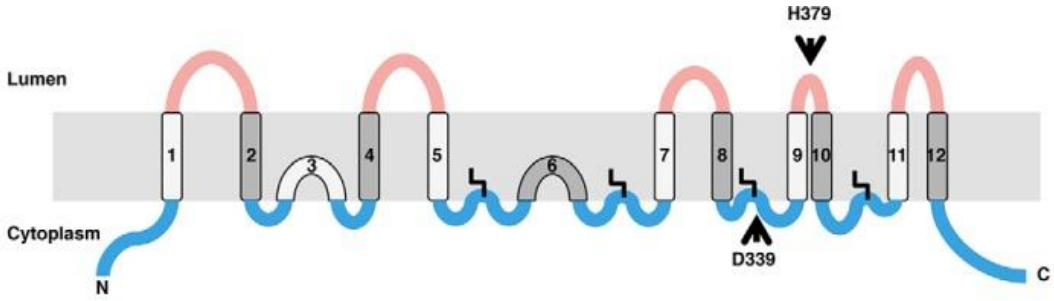

Figure 2 - Model for the membrane topology of HHAT. HHAT is comprised of ten TMD and two reentrant loops, the TMD3 and 6. Both N- and C-terminal predicted to be localized in the cytosol. The critical His-379 and Asp-339 residues (indicated by arrows) are positioned on opposite sides of the membrane. The cytosolic loops containing cysteines that are palmitoylated display the fatty acid modification as a bent line. Image adapted from Konitsiotis *et al.*, 2015.

Importantly, some of the cytosolic loops that exist between the TMD and the two re-entrant loops of mammalian HHAT are supposedly stabilized by palmitoylation of Cys residues at least in four different positions (Konitsiotis et al 2015). *S. cerevisiae* Gup1 and Gup2 and *C. albicans* Gup1 present a partially hydrophobic region, located between TMD 7 and 8 (using the TMD prediction engine available at http://www.cbs.dtu.dk/services/TMHMM/). The correspondent sequence aligns perfectly with the sequence from the *M. musculus* HHAT comprising both MBOAT signature (*M. musculus* Asp^{339} and His^{374}), showing several additional conserved amino acid residues. *In silico* palmitoylation prediction of Gup1 and Gup2 amino acid sequence (using the prediction engine CSS-Palm available at http://csspalm.biocuckoo.org/online.php) indicates the possible palmitoylation of a fully conserved cysteine residue in that region (Fig. 1): *S. cerevisiae* Gup1 Cys^{396} and Gup2 Cys^{445}; *C. albicans* Gup1 Cys^{417}; *M. musculus* HHAT Cys^{324} and HHATL Cys^{326}; *H. sapiens* HHAT Cys^{325} and HHATL Cys^{326}; and *D. melanogaster* RASP Cys^{326}. This degree of conservation, in addition to the possibility that a palmitoylation is involved, suggests that Gup proteins might require the MBOAT signature residues to be embedded in or stabilize at the surface of the membrane.

GUP1 and *GUP2* Expression in Yeast

According to the engine that analyses the yeast genome for transcription factors (TF) recognition sequences YEASTRACT (http://www.yeastract.com/), the 1,000 bp

upstream region of *GUP2* displays consensus sequences for 65 TF, and *GUP1* for 19 TF (Table 2). These include major players in yeast transcription control, like (i) the general stress regulators Msn2 and Msn4, (ii) the glucose-repression controller Mig1, (iii) Ste12 from pheromone response and mating, and (iv) Gcn4 from nitrogen-associated regulation. Three TFs are predicted to regulate both genes promoters identically (Tec1, Mig3 and Rap1), while one, Ash1, is predicted to activate *GUP1* and inhibit *GUP2* transcription (Table 2).

The presence of numerous TF consensus recognition sequences in the promoters of yeast *GUP1*/2 could reflect a complex transcription regulation. *GUP1* and *GUP2* are though almost invariantly expressed in several culture conditions, leading to suggest that the expression of these genes might be constitutive (Oliveira and Lucas, 2004). This is the case for cells actively growing on glucose (repression conditions - fermentation) and on glycerol or ethanol (de-repression conditions - respiration) (Oliveira and Lucas, 2004), as well as in cells osmotically stressed with high amounts of salt (Posas *et al.*, 2000; Oliveira and Lucas, 2004), or subjected to osmotic shock using high amounts of sorbitol (Rep *et al.*, 2000). Still, *GUP1* transcription varies, duplicating upon NaCl shock (Yale and Bohnert, 2001) in opposition to sorbitol shock (Rep *et al.*, 2000), and decreasing greatly after rapamycin treatment or amino acid deprivation (Hardwick *et al.*, 1999).

GUP2 was found to be generally less expressed than *GUP1* (Oliveira and Lucas, 2004). The lower expression of *GUP2* would be in agreement with the fact that Δ*gup2* mutants do not present any marked phenotype in response to changes in carbon source (glucose/glycerol), or in response to stress (salts, sorbitol, ethanol, weak acids and high temperature). Still *GUP2* expression increases considerably upon ultraviolet irradiation (Dardalhon *et al.*, 2007), leading the authors to suggest that Gup2 could act on the Early Genotoxic Response. Over-expression of *GUP1* induces the up-regulation of several genes coding for proteins resident in the ER and Golgi, from the secretory pathway and lipid synthesis (Bleve *et al.*, 2011). Kar2, a key protein from the Unfolded Protein Response (UPR) signalling pathway, stands out for its 24-fold increased expression.

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GUP1 further induces proliferation of membranous cisternal structures that are physically separated from each other (Bleve *et al.*, 2011). This phenomenon is dependent on Ire1, another protein from the UPR pathway. ER, Golgi and itinerant proteins are present in these structures. Gup1 was also found in those proliferated membrane structures, and showed to be able to move within the proliferated membrane structures diffusing in and out (Bleve *et al.*, 2011). Remarkably, the production of these membrane structures is dependent on the His^{447} MBOAT signature amino acid (Bleve *et al.*, 2011).

Table 2 - Documented transcription factors that function as regulators of Gup1 and Gup2. Activ.: activator; Inhib.: inhibitor. Table published in Lucas *et al.,* 2016.

Predicted Function in *GUP1* and *GUP2* Transcription Regulation											
GUP1			***GUP2***				**Identical Regulation**			**Opposite Regulation**	
Activ.	**Inhib.**	**Both**	**Activ.**		**Inhib.**	**Both**	**Activ.**	**Inhib.**	**Both**	**Activ. *GUP1* + Inhib. *GUP2***	**Inhib. *GUP1* + Activ. *GUP2***
Ash1	Abf1	Rap1	Ecm22	Sfp1	Ace2	Aft1	Tec1	Mig3	Rap1	Ash1	none
Met4	Cac2	Spt23	Gat4	Snf2	Ash1	Cbf1					
Tec1	Cup9	Yrm1	Gcr2	Snf6	Bas1	Cin5					
	Gal4		Gis1	Sok2	Fhl1	Plm2					
	Hir3		Gln3	Spt10	Gcn4	Pho4					
	Mig3		Gzf3	Spt2	Gcr2	Rap1					
	Sfp1		Hap5	Ste12	Mig3	Rfx1					
	Sum1		Hda1	Sut1	Msn4	Sko1					
	Uls1		Hms1	Swi3	Rif2	Xbp1					
			Isw2	Taf14	Set2	Yap6					
			Mbp1	Tec1	Swi5	Msn2					
			Mcm1	Tup1	Tbf1	Swi4					
			Mga1	Uls1							
			Mig1	Uc2							
			Mot3	Urc2							
			Nrg1	Xbp1							
			Rgm1	Yhp1							
			Rox1	Yox1							

Gup1 and Gup2 Subcellular Localisation

The Gup1 protein co-localizes with markers of the plasma membrane, the endoplasmic reticulum (ER) and the mitochondria (Holst *et al.*, 2000). The more prominent localizations of Gup1 appear to be at the plasma membrane and ER, as revealed by GFP tagging of either N- or -C-terminal (Bleve *et al.*, 2005). On the other hand, although Gup2 is predicted to be also a membrane protein, its *in vivo* localization was never studied. The plasma membrane localization of Gup1 is dependent on the secretory pathway proteins Sec6-4 (Bleve *et al.*, 2005). Moreover, Gup1 is steadily internalized in an End3-dependent manner upon sudden shift from glycerol to glucose (Bleve *et al.*, 2005; Ferreira *et al.*, 2005). The fact that Gup1 is retrieved from the plasma membrane upon glycerol-to-glucose transition establishes a connection with the regulation by carbon source, which can be associated directly or indirectly with glycerol considering the very first results that yielded the discovery of *GUP* genes, and which nature remains for the time being unknown.

Yeast Phenotypes Associated with the Deletion of *GUP1*

In yeast, *GUP1* gene is a very pleiotropic gene that influences a great deal of apparently unrelated cellular processes. Its deletion is associated with a high number and diversity of biological functions detailed below, that include important key features of yeast life such as polarity establishment, secretory/endocytic pathway functionality, vacuole morphology and wall and membrane composition, structure and maintenance (Fig. 3). Phenotypes underlying death, morphogenesis and differentiation are also included. The major players implicated in those phenotypes is showed in Fig. 4, from which the HOG (High Osmolarity Glycerol) pathway, the CWI/PKC (Cell Wall Integrity) pathway and the crosstalk between HOG, CWI, the complex lipids (LCBs) and TORC1/2 (Target of rapamycin 1/2 complex) signalling pathways, are stressed.

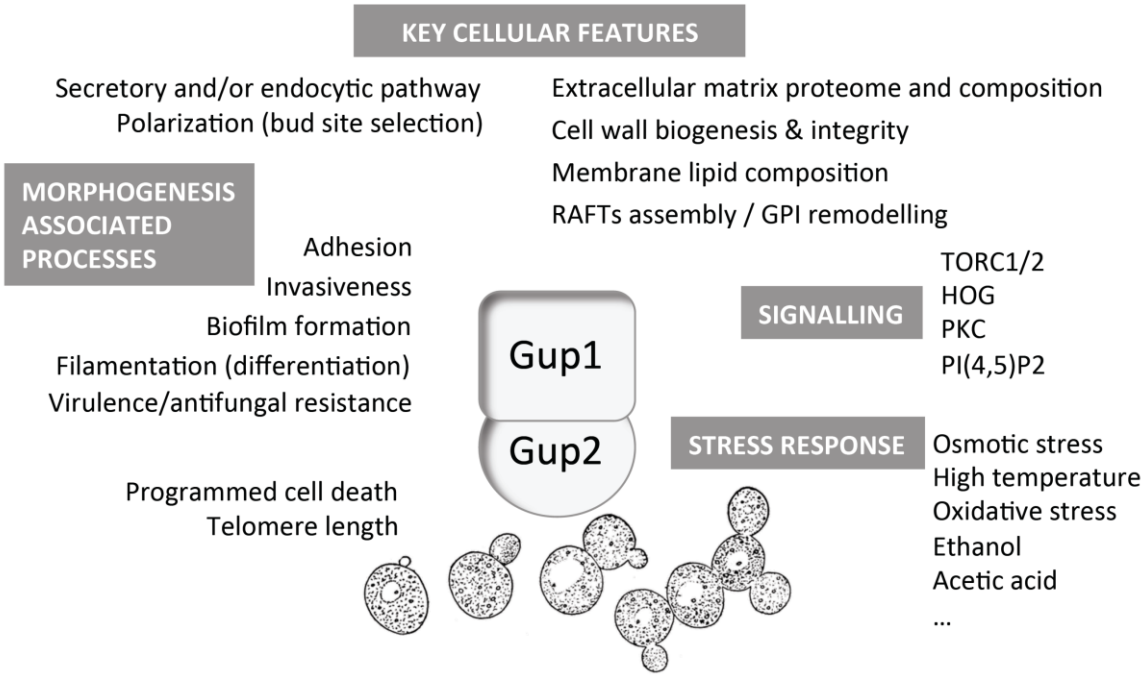

Figure 3 - Phenotypes and biological processes associated to the Gup proteins in yeasts (*S. cerevisiae* and *C. albicans*). Image published in Lucas *et al.*, 2016.

Phenotypes Emerging from Genome-Wide Yeast Screenings

The extensive list of phenotypes associated with the deletion of *GUP1* comprises many that were obtained in genome-wide screening. These include cytoskeleton polarization and bud site selection patterns (Ni and Snyder, 2001), secretory and endocytic pathways (Bonangelino *et al.*, 2002), vacuole morphology (Bonangelino *et al.*, 2002) and anaerobic growth (Reiner *et al.*, 2006). Other high throughput studies cannot be presently rationalized, like the one mentioning Gup1 as an important protein in the maintenance of proper telomere length (Askree *et al.*, 2004). Importantly, many of these studies show the resistance/sensitivity of the Δ*gup1* mutant to pharmaceutical drugs such as the anti-tumoral Imatinib (dos Santos and Sá-Correia, 2009), the cytostatic Cisplatin (Liao *et al.*, 2007), the anti-inflammatory Ibuprofen (Tucker and Fields, 2004), the immunosuppressant FK506 (Viladevall *et al.*, 2004) and the antimicrobial Thymol (Darvishi *et al.*, 2013), among others. This large list, by itself, corroborates an equally large complexity of Gup1 functions.

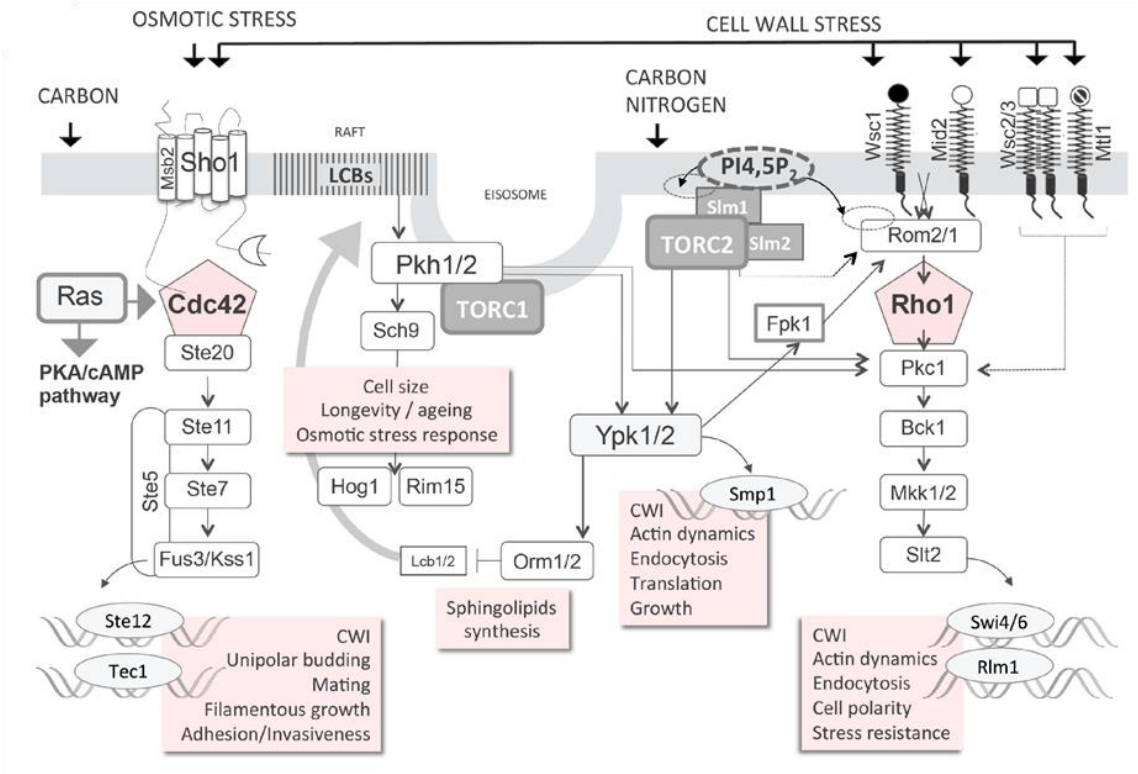

Figure 4 - Major players implicated in the phenotypes associated with the deletion of *GUP1* in yeast. The Sho1 downward cascade belongs to the HOG (High Osmolarity Glycerol) pathway, and the Rom/Rho cascade constitutes the CWI/PKC (Cell Wall Integrity) pathway. The crosstalk between HOG, PKC, the complex lipids (LCBs) and TORC1/2 signaling pathways relevant for *GUP1* associated phenotypes is shown. Plain arrows indicate established interactions; dashed arrows indicate possible interactions. Pentagons refer to nodal proteins exerting multiple signaling and affecting positively or negatively many other proteins. Image published in Lucas *et al.*, 2016.

Cell Wall Integrity and Biogenesis

As mentioned above, Δ*gup1* is very sensitive to high temperature (Oelkers *et al.*, 2000; Ferreira *et al.*, 2006). Generally, the sensitivity of yeast to high temperature is mostly correlated with major defects in the cell wall. Consistently, the deletion of *GUP1* in *S. cerevisiae* causes an increase in the cell wall amounts of β-1,3-glucans (+25%) (Ferreira *et al.*, 2006) and chitin (+90%) (Lesage *et al.*, 2005; Ferreira *et al.*, 2006), and a decrease in the amount of mannoproteins (−70%) (Ferreira *et al.*, 2006). These large differences are responsible for a deficient wall morphology, and increased sensitivity to wall-perturbing agents, such as Calcofluor White (CFW), Congo Red (CR) and caffeine, as well as to cell wall-degrading enzymes, like zymolyase and lyticase (Ferreira *et al.*, 2006). The Δ*gup1* mutant also displays a sedimentation/aggregation phenotype that does not account for any of the described flocculation phenotypes

(Ferreira *et al.*, 2006). In spite of these defects, the CWI/PKC pathway is signalling normally in the Δ*gup1* mutant, as evidenced by MAPK (Slt2) dually phosphorylated state (de Nobel *et al.*, 2000) after induction of the pathway by hypo-osmotic shock (Ferreira *et al.*, 2006).

The CWI/PKC pathway controls not only the biogenesis of cell wall constituents and their remodelling, but also the associated organization of the actin cytoskeleton and secretory pathway (reviewed by Levin (2011)). *S. cerevisiae* only has one protein kinase C (Pkc1) that responds to the G-protein Rho1 (Fig. 5). Rho1 activates Pkc1, besides other effectors, which allows the coordinated control of the actin cytoskeleton, exocytosis, membrane fluidity and the synthesis of wall glucans through transcription regulation in response to nutrients and stress (Lockshon *et al.*, 2012). Rho1 in turn is activated by Rom2/1 GDP/GTP exchange factors. These respond directly at least to a couple of wall stress sensors, Wsc1 and Mid2, and are further activated by the ligation of PI(4,5)P2 (phosphoinositol 4,5-biphosphate) and/or of Slm1/2 proteins. These are in turn targets for TORC2 phosphorylation (Audhya *et al.*, 2004). TORC2 also directly activates Pkc1, inducing the PKC pathway response. Importantly, Rho1 and consequently the PKC pathway are also activated by the α-factor plasma membrane receptor Ste2, a physical partner of Gup2 according to Saccharomyces Genome Database.

TORC2 is redundant with TORC1 in the control of major cellular functions, but the cell wall composition and integrity, as well as the polarization of the actin cytoskeleton associated with cell growth, division and morphogenesis, are only dependent on TORC2. Many of the cellular functions controlled by TORC2 are defective in the absence of *GUP1*. Besides wall damage-associated phenotypes, the most striking of these is the altered polarity during division exhibited by Δ*gup1* mutant (Ni and Snyder, 2001; Casamayor and Snyder, 2002). The Δ*gup1* mutant was found to display profoundly aberrant bud site selection (Ni and Snyder, 2001; Casamayor and Snyder, 2002). This implies a large defect on the proper establishment of cytoskeleton polarity (Casamayor and Snyder, 2002; Wu *et al.*, 2013), which is known to depend on Rho1/Pkc1 signalling (Perez and Rincón, 2010). It has been proposed that Rho1, whose activation is regulated by Tor2, binds to and activates Pkc1 to promote actin polarization. It could be as well dependent on other pathways associated with the

multiple roles of the signalling node Cdc42 (Martin, 2015), including the Sho1 branch of the HOG pathway (Raitt *et al.*, 2000).

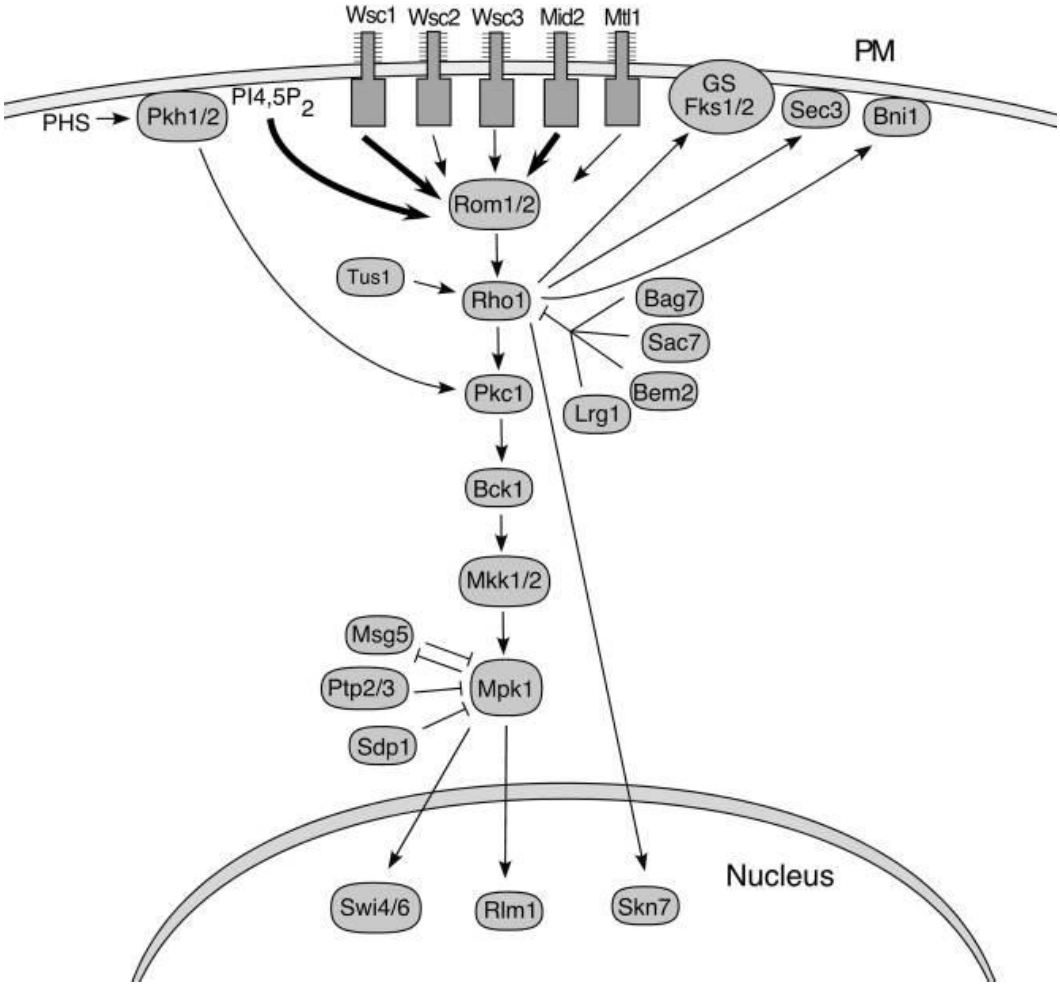

Figure 5 - Schematic representation of the CWI/PKC signaling pathway. Signals are initiated at the plasma membrane through the cell surface sensors Wsc12/3, Mid2, and Mtl1. Together with PI4,5P2, which recruits the Rom1/2 GEFs to the plasma membrane, the sensors stimulate nucleotide exchange on Rho1. Rho1 activates several effectors, including the Pkc1-MAP kinase cascade. The MAP kinase cascade, which is comprised of Bck1, Mkk1/2, and Mpk1 (Slt2), is activated by Pkc1. Two transcription factors, Rlm1 and the SBF complex (Swi4/Swi6), are the ultimate targets of this pathway. Image from Levin, 2005

TORC1, on the other hand, is primarily involved on regulating nutrient sensing and biosynthesis, ribosome biogenesis and translation, mitochondrial function, lifespan, and autophagy-related processes (for a review see Loewith and Hall (2011)). In addition, TORC1 regulates sphingolipid metabolism by inhibiting the synthesis of complex sphingolipids (Shimobayashi *et al.*, 2013). Even though TOR proteins are very similar phosphatidylinositol kinase (PIK)-related kinases, only TORC1, and not TORC2, is

sensitive to inhibition by caffeine or the immunosuppressor rapamycin (Wanke *et al.*, 2008; Loewith and Hall, 2011). The Δ*gup1* mutant, both alone and in combination with Δ*gup2*, is resistant to rapamycin (Ferreira, 2005). On the other hand, Δ*gup1* is equally sensitive to caffeine as the *wt* strain, but, unlike this last strain, its sensitivity is remediable with sorbitol (Ferreira *et al.*, 2006). The mechanisms of action of caffeine and rapamycin are poorly understood. Both induce Rho1 activation and depend on this induction to inactivate the TORC1 pathway (Yan *et al.*, 2012). Caffeine induces the activation of Slt2 (Mpk1) in a Tor1/Rom2 dependent manner and elicits a transient decrease in the intracellular levels of cAMP, concomitantly inhibiting the Ras/cAMP pathway (Kuranda *et al.*, 2006). Therefore, the activation of Pkc1 cascade by caffeine is mediated by the inhibition of Tor1 signalling, being independent of the CWI/PKC pathway main sensors Wsc1 and Mid2 (Kuranda *et al.*, 2006). Rapamycin, on the other hand, represses rRNA transcription and induces arrest of cell cycle at G1. Furthermore, it causes glycogen accumulation, sporulation and autophagy (Hardwick *et al.*, 1999). These effects demand the complexation of rapamycin with Fpr1 and subsequently with Tor1 (Lorenz and Heitman, 1995). The way in which Gup1 might be involved remains obscure. Bearing in mind the defects that the deletion of *GUP1* causes in the cell wall (Ferreira *et al.*, 2006) and membrane (Oelkers *et al.*, 2000; Ferreira and Lucas, 2008), and in the endocytosis process (Bonangelino *et al.*, 2002), the cause of resistance to rapamycin could be the inability of the drug to enter the cell. Rapamycin is practically insoluble in water and poorly soluble in ethanol or glycerol (Simamora *et al.*, 2001) and has a large molecular mass (≈900 Da), which suggests it enters by endocytose.

Yeast TOR pathways respond mainly to carbon and nitrogen availability (Staschke *et al.*, 2010). *In silico* analysis of the promoter regions of both *GUP1* and *GUP2* unveiled a series of putative regulatory sequences, from which stands out the presence of repeated consensus sequences for Gcn4, a major transcription factor that responds to amino acid starvation and general amino acid control in the dependence of TORC1 (Valenzuela *et al.*, 2001). Accordingly, wild-type strain and Δ*gcn4* mutant expressing lacZ reporter constructions for *GUP1* and *GUP2* promoter regions showed that β-galactosidase activity depended on Gcn4 for full expression of the *GUP1* but not *GUP2* (Ferreira, 2005). The Gcn4 dependence for *GUP1* expression suggests that TORC1 could be regulating Gup1.

High Osmolarity Glycerol Pathway

In spite of the *GUP1* deletion, the CWI/PKC pathway is working properly at the level of Slt2 phosphorylation when stimulated by hypotonic shock (Ferreira *et al.*, 2006). Nevertheless, the wall of Δ*gup1* mutant is severely affected. Two other pathways contribute to cell wall integrity and remodelling, the Sho1 branch of the HOG pathway, and the Long-Chain Base (LCB)/sphingolipids YPK pathway (this last one will be discussed in the next section).

HOG stands for High Osmolarity Glycerol, a name derived from the production and accumulation of the yeast osmolyte glycerol in response to high osmotic stress (extensively reviewed by Hohmann (2002)) (Fig. 6). The HOG pathway controls many other processes besides glycerol production in response to stress, such as polarity, adhesion and invasiveness, filamentation (*i.e.* differentiation), mating and cell wall biogenesis (reviewed by Hohmann (2015)). This pathway is functionally equivalent to the mammalian Wnt/p38 stress responsive MAPK pathway (Gordon and Nusse, 2006; Cuadrado and Nebreda, 2010) that is responsible for the control of growth/proliferation, i.e. cell cycle progression, as well as filamentation, adhesion, migration and apoptosis, in response to growth factors, cytoquines and stress (Gallo and Johnson, 2002). The HOG pathway comprises the upstream Sln1 and Sho1 branches, both of which activate the Hog1 MAPK. (Fig. 6). The Sho1 branch employs two related, but distinct, signalling mechanisms, the Hkr1 and Msb2 sub-branches. A signal emanating from either branch converges on Pbs2, which is the specific activator of the Hog1 MAPK (Brewster *et al.*, 1993; Maeda *et al.*, 1994, 1995). Sln1 branch transmits a signal via the Sln1-Ypd1-Ssk1 phosphorylate cascade (Posas *et al.*, 1996; Reiser *et al.*, 2003). In contrast, Sho1 branch functions in an exceptionally complex manner (Tatebayashi *et al.*, 2015). It binds to effectors that deliver different responses, Hkr1, that activates Hog1 through the Ste20–Ste11–Pbs2–Hog1 kinase cascade, and Msb2, that functionally interacts with the scaffold protein Bem1 to activate Ste20 (Tanaka *et al.*, 2014; Tatebayashi *et al.*, 2015; Nishimura *et al.*, 2016).

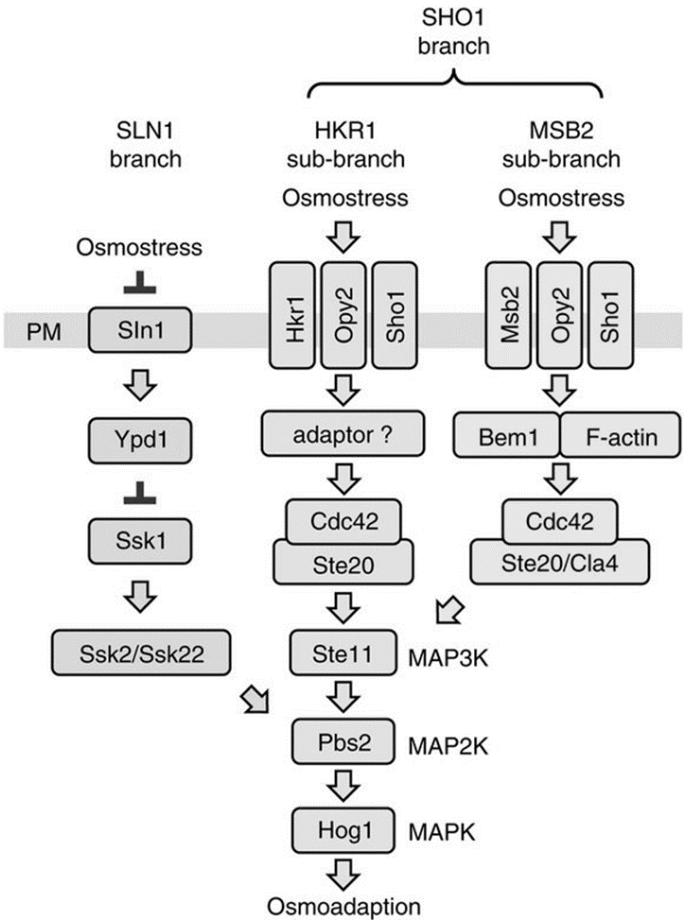

Figure 6 - Schematic representation of the HOG signaling pathway. The HOG pathway comprises the upstream Sln1 and Sho1 branches. The Sho1 branch employs two related, but distinct, signaling mechanisms, the Hkr1 and Msb2 sub-branches. Both branches activate the Hog1 MAPK signaling cascade that eventually culminate in the Hog1 activation. Image from Tatebayashi et al., 2015.

The strains without *GUP1* are unable to grow under high osmotic stress (Holst *et al.*, 2000; Ferreira *et al.*, 2006). Hyperosmotic stress stimulates mainly the HOG pathway but also the CWI/PKC pathway (Rodríguez-Peña *et al.*, 2010), a fact that is not surprising since the adaptation to high osmotic stress implicates changes in cell volume and turgor that demand remodelling of the cell wall. However, and as previously mentioned, CWI/PKC pathway is working properly in Δ*gup1* mutant despite the severely affected cell wall (Ferreira *et al.*, 2006). This could indicate that this mutant has a malfunction of the HOG rather than CWI/PKC pathway, and a concomitant deficient production and/or accumulation of glycerol. The simultaneous activation of HOG and CWI/PKC pathways can also result from other stimuli, such as high temperature as mentioned above (Winkler *et al.*, 2002), and the action of zymolyase, an

enzyme cocktail with β1,3-glucanase activity (Alonso-Monge *et al.*, 2001; Bermejo *et al.*, 2008). Δ*gup1* is extremely sensitive to both stimuli (Ferreira *et al.*, 2006). The effect of zymolyase is particularly severe, namely in comparison with lyticase, another wall disrupting enzyme (Ferreira *et al.*, 2006). Still, this severity could be the consequence of the increased percentage of β1,3-glucans in the mutant cell wall. Another evidence towards the malfunction of HOG rather than CWI/PKC pathway was inferred from the pronounced phenotype of Δ*gup1* mutant in the presence of CFW (Ferreira *et al.*, 2006), compared to CR that caused a negligible defect (Faria-Oliveira, 2013). While some insults to the cell wall like CR only trigger CWI/PKC (García *et al.*, 2004; Kuranda *et al.*, 2006), others like CFW trigger both HOG and CWI/PKC pathways (Alonso-Monge *et al.*, 2001; Winkler *et al.*, 2002; Bermejo *et al.*, 2008; Rodríguez-Peña *et al.*, 2010). In spite of the intricate interplay between both pathways (reviewed by Rodríguez-Peña *et al.* (2010)) the phenotypes caused by *GUP1* deletion associated with osmoregulation (Holst *et al.*, 2000) and the cell wall (Ferreira *et al.*, 2006) are consistent with a relation of Gup1 with both pathways (Lucas *et al.*, 2016).

Plasma Membrane Composition and Associated Signalling

Gup1 is implicated in several membrane and lipids associated phenotypes (Oelkers *et al.*, 2000; Bosson *et al.*, 2006; Reiner *et al.*, 2006; Ferreira and Lucas, 2008). Among these, the lipid membrane composition of *Δgup1* cells exhibits a reduced content of phospholipids and elevated levels of diacylglycerols (DAG) and triacylglycerols (TAG) (Oelkers *et al.*, 2000) (Fig. 7). Moreover, Gup1 interferes in sterol and sphingolipids synthesis, as evidenced by increased and decreased resistance of the mutant to sterol and sphingolipids inhibitors, respectively (Ferreira and Lucas, 2008). Fatty acids synthesis, on the other hand, appears to remain normal (Ferreira and Lucas, 2008). Gup1 also affects lipid rafts integrity and assembly (Ferreira and Lucas, 2008). Similarly to *S. cerevisiae*, deletion of *GUP1* in *C. albicans* also modifies ergosterol distribution at the level of plasma membrane (Ferreira *et al.*, 2010). Among various lipids of yeast membranes, ergosterol has been used as target of the most common antifungals, like polyenes and azoles (Sanglard *et al.*, 2003; Pemán *et al.*, 2009). Accordingly, *C. albicans caΔgup1* null mutant displayed increased resistance ergosterol synthesis inhibitors such as fluconazole, ketoconazole and clotrimazole (Ferreira *et al.*, 2010).

Moreover, the uptake of sterols is impaired in Δ*gup1* mutant (Reiner *et al.*, 2006). Yeasts are facultative anaerobic organisms that can efficiently endure environments with virtually inexistent oxygen. In these conditions, the yeast cell does not synthesize sterols. Instead, it must take up sterols from the environment, using the Pdr11 ATP-binding cassette (ABC multidrug transporter (Li and Prinz, 2004). This is most possibly the cause for Δ*gup1* inability to grow in anaerobic conditions (Reiner *et al.*, 2006; Samanfar *et al.*, 2013).

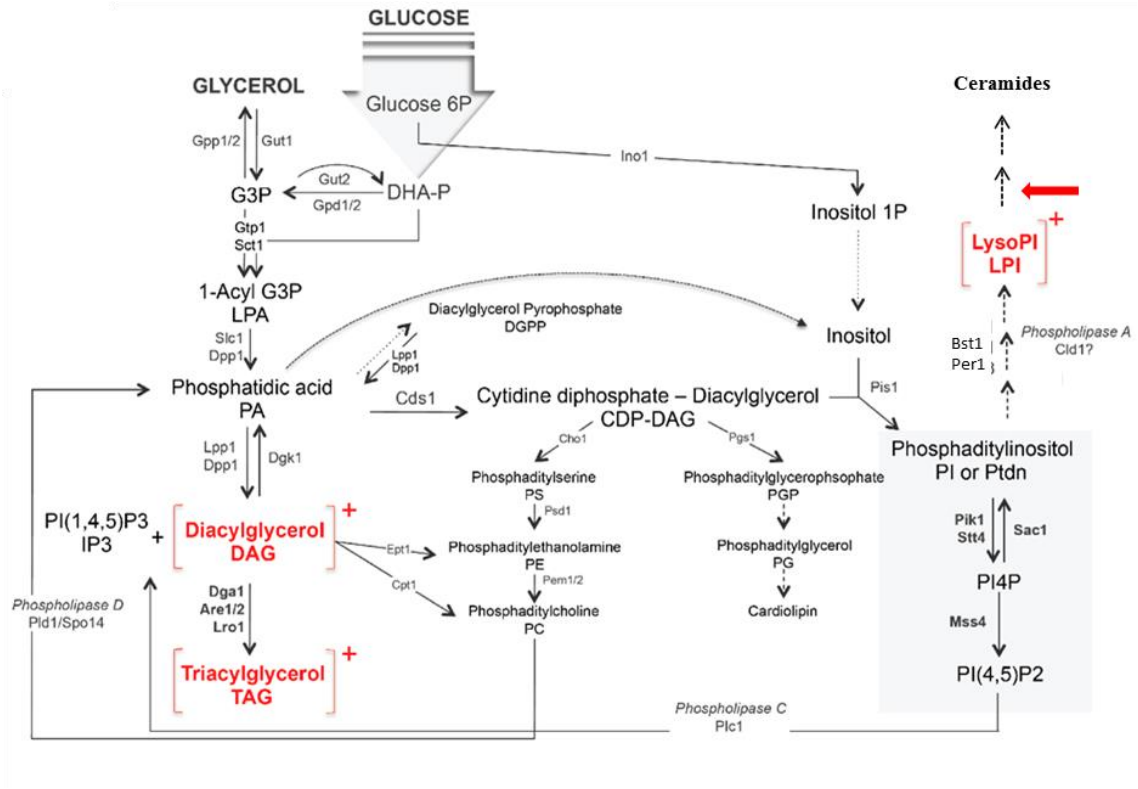

Figure 7 - Key lipid synthesis pathways from *S. cerevisiae*. In red are shown the types of lipids which amounts are affected by the *GUP1* deletion. Image published in Lucas *et al.*, 2016.

In yeast, the signalling pathway associated with complex lipids is the LCB/sphingolipid YPK pathway (also referred as TORC2-Ypk1 pathway) (Fig 8). The activation of Ypk1 is dependent on phosphorylation by Pkh1/2 kinases and are activated in response to changes in LCBs (Friant *et al.*, 2001; Roelants *et al.*, 2002, 2011). Ypk1 activity can be substantially increased by a further phosphorylation by TORC2 (Kamada *et al.*, 2005; Niles *et al.*, 2012). Besides the sphingolipid depletion, certain membrane stress conditions increase TORC2 activity and consequently activate Ypk1, such as heat shock (Sun *et al.*, 2012), and hypoosmotic stress (Berchtold *et al.*, 2012). In these cases,

Ypk1 phosphorylates Orm1/2, Lac1 and Lag1 that ultimately culminate in the stimulation the biosynthesis of complex sphingolipid to re-establish the correspondent levels (Roelants *et al.*, 2011; Muir *et al.*, 2014).

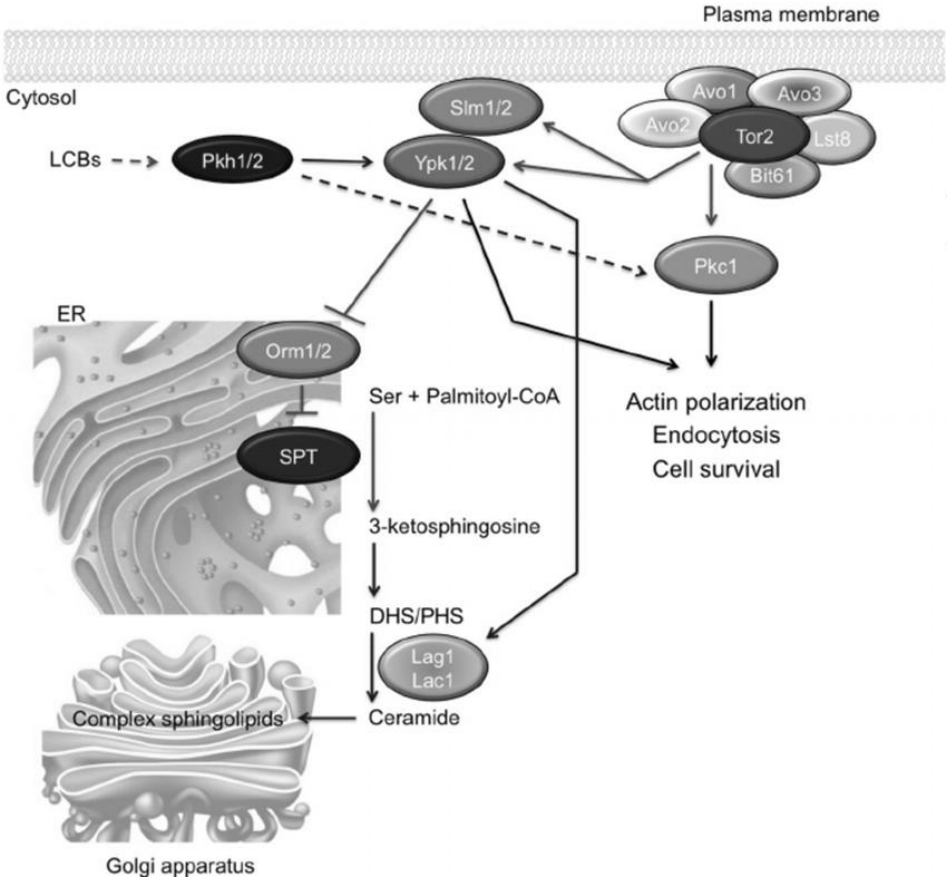

Figure 8 - TORC2/Ypk1/2 signaling in yeast. The activation of Ypk1/2 is dependent on phosphorylation by Pkh1/2 kinases, which in turn is activated by LCBs, and/or by phosphorylation by TORC2. The active Ypk1/2 kinase phosphorylates Orm1/2p, Lag1p and Lac1p, thus stimulating the de novo biosynthesis of complex sphingolipids upon sphingolipid depletion or mechanical stress. Image from Teixeira and Costa, 2016.

Other stress conditions, such as hyperosmotic stress, diminish TORC2-Ypk1 activity (Muir *et al.*, 2015). Down-regulation of TORC2-Ypk1 signalling allows cell survival under high osmotic stress independently of the activation of HOG pathway (Muir *et al.*, 2015). TORC2-Ypk1 signalling operates through closing of the glycerol channel Fps1, by blocking its phosphorylation by Ypk1 (Muir *et al.*, 2015). Fps1 is essential for yeast to survive hyperosmotic stress. Its closing prevents glycerol efflux promoting intracellular retention of this essential osmolyte (Luyten *et al.*, 1995; Tamás *et al.*, 1999). Moreover, Ypk1/2, besides phosphorylating Fps1 thereby promoting the opening

of the channel, also inhibits Gpd1 (cytosolic glycerol 3 phosphate dehydrogenase), the enzyme responsible for deviating the glycolytic flux towards glycerol production (Lee *et al.*, 2013). The need for a TORC1 and Ypk1/2 control of the glycerol production and retention strengthens the notion firstly suggested by Siderius *et al.*, (2000) that glycerol, could be also important in maintaining the signalling competent state of cells. The defects caused by *GUP1* deletion include glycerol active uptake (Holst *et al.*, 2000) and sphingolipids metabolism (Oelkers *et al.*, 2000; Ferreira and Lucas, 2008), suggesting that Gup1 protein could have a related role.

LCB/sphingolipids assemble Detergent-Resistant Microdomains (DRM) in the plasma membrane, also known as lipid rafts due to their particular molecular and physicochemical properties. These properties derive from the predominantly saturated hydrocarbon tails of the sphingolipids and the high concentration of sterols: cholesterol in animal cells (van Meer, 1989) and ergosterol in yeasts (Bagnat *et al.*, 2000; Jacobson *et al.*, 2007). In yeast, as in mammalian cells, lipid rafts participate in protein sorting, dynamically including or excluding proteins, and thus contributing to the function of transporters, pumps, sensors, receptors and G-proteins (Bagnat and Simons, 2002; Mollinedo, 2012; Spira *et al.*, 2012). Importantly, the components of lipid rafts are also actively involved in signalling and in stabilization of actin cytoskeleton–membrane interactions and therefore polarity (Bagnat and Simons, 2002; Spira *et al.*, 2012). In yeast, many proteins are known to functionally localize in lipid rafts forming a complex patchwork (Spira *et al.*, 2012), which biogenesis is still poorly known. The first proteins found in yeast membrane microdomains were the H^+-ATPase pump Pma1 (Bagnat *et al.*, 2001) and the amino acid permease Can1 (Malínská *et al.*, 2003). Presently, many yeast proteins are known to differentially localize into lipid rafts (Spira *et al.*, 2012). These include permeases involved in the transport of drugs, ions, water and glycerol homeostasis, this last through Fps1 channel. Rafts also include proteins involved in sugar or nitrogen transport/sensing, signalling, stress response, cell wall metabolism, flocculation, bud site selection and eisosome formation. Some of these proteins are GPI-anchored, like the β1,3-glucanosyltransferase Gas1 (Bagnat *et al.*, 2000), while others are prenylated, or farnesylated and palmitoylated like Ras2 (Kuroda *et al.*, 1993), but most just have immersed in the lipids their transmembrane domains (see Spira *et al.*, 2012 for a comprehensive list).

Different types of membrane lipid microdomains were identified and designated on the basis of the proteins they harbour: Membrane Compartments occupied by Pma1 (MCP) (Bagnat *et al.*, 2001), Can1 (MCC) (Malínská *et al.*, 2003) or TORC2 (MCT) (Berchtold and Walther, 2009). Δ*gup1* mutant is affected in lipid rafts integrity and assembly (Ferreira and Lucas, 2008). This was evidenced by the even, not punctate, distribution of sterols in the mutant plasma membrane, as well as the 40% lower amounts of DRM recovered (Ferreira and Lucas, 2008). Additionally, Pma1 (the H^+-pump known to reside in lipid rafts, specifically MCPs) is absent from Δ*gup1* DRMs, and the amounts of Gas1 (the most abundant yeast GPI-anchored protein) are residual, suggesting that rafts were unselectively affected by *GUP1* deletion (Ferreira and Lucas, 2008). These proteins were later found to be greatly secreted in the Δ*gup1* mutant (Faria-Oliveira, 2013).

Yeast lipid rafts assembly takes place in the ER, subsequently stepping into the Golgi (Bagnat *et al.*, 2000). Moreover, MCPs and MCCs are likely to segregate differently, since Pma1 is only incorporated into the rafts outside the ER (Bagnat *et al.*, 2001). ER resident proteins targeted to sphingolipids/ergosterol-poor membranes, such as the vacuole membrane, are not involved in rafts assembling (Bagnat and Simons, 2002). Therefore, the defect in rafts assembly generated by *GUP1* deletion must occur in the ER (one of Gup1 localizations) and may induce the segregation of specific membrane/protein assemblages towards their correct location. Another group of results concurs for the implication of the Gup1 protein in the secretory/endocytic pathways (Bonangelino *et al.*, 2002). Proteins for degradation are addressed to the vacuole by the same pathway as carboxypeptidase Y (CPY), through the late endosome (reviewed by Odorizzi *et al.*, 1998). *GUP1* was identified as one of the genes required for efficient sorting of CPY to the vacuole, as well as for the maintenance of this organelle regular size and morphology (Bonangelino *et al.*, 2002). Its deletion also causes the excessive secretion of the ER-resident protein Pdi1 (Ferreira *et al.*, 2006), and the GPI-anchored β1,3-glucanosyltransferase Gas1 (Ferreira and Lucas, 2008).

As mentioned above, the deletion of *GUP1* affects sterols and sphingolipids synthesis, impairs sterols uptake, and causes changes in the regular concentrations of major lipid types (Fig. 7). Still, Gup1 is not recognized as an enzyme from lipid biosynthesis pathways. In this regard, Bosson and co-workers proposed the involvement

of Gup1 in the GPI synthesis/remodelling process (Bosson *et al.*, 2006), which occurs at the level of the ER in *S. cerevisiae* (Bosson *et al.*, 2009; Fujita *et al.*, 2011).

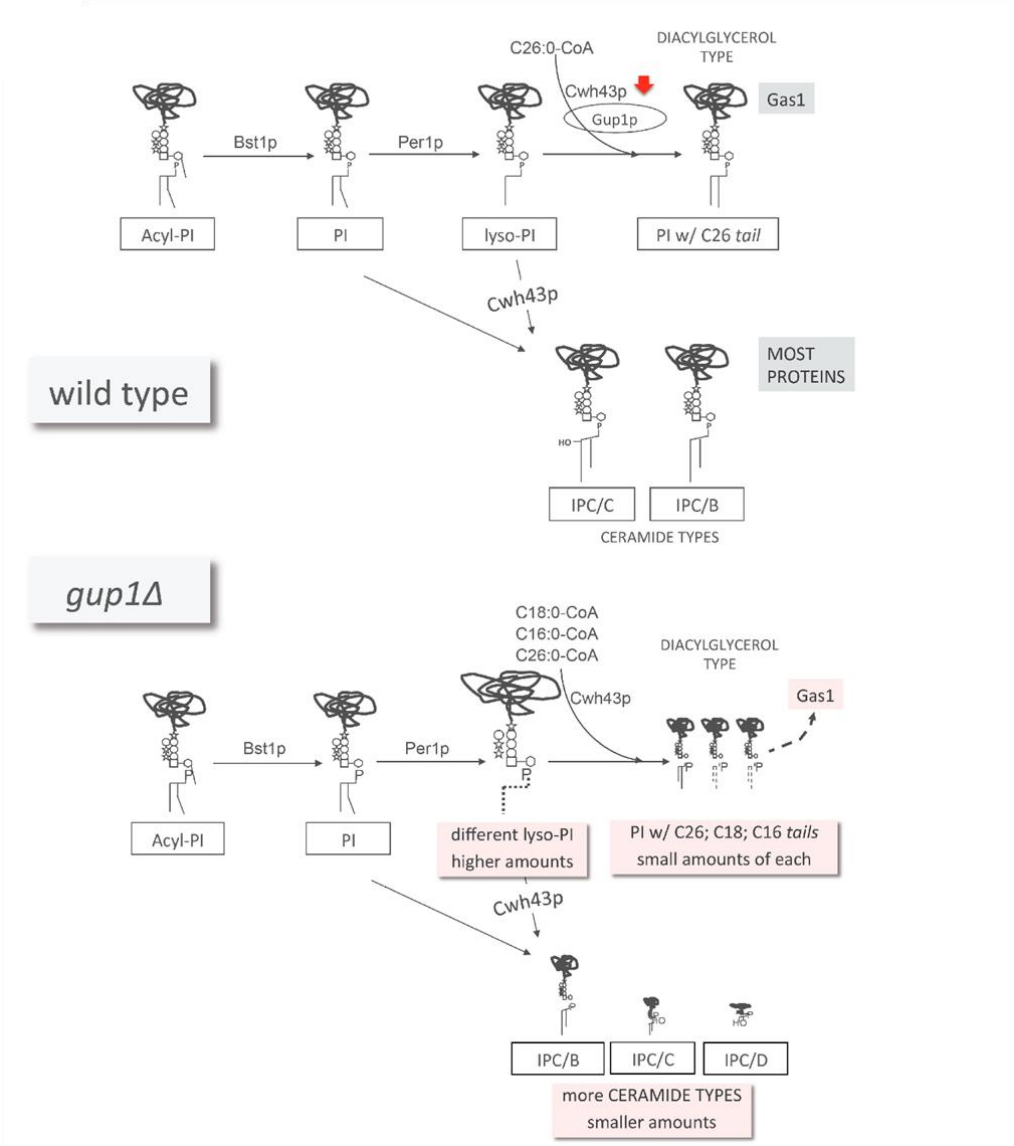

Figure 9 - Steps in GPI anchor synthesis occurring in *S. cerevisiae wt* strain and in Δ*gup1* mutant. Gas1 is an example of a GPI anchored protein that is found excessively liberated into the medium in the mutant cultures. Red arrows indicate the step in which Gup1 was suggested to act as an acyltransferase. GPI anchor backbone is composed of mannose (O), glucosamine (☆) and ethanolamine-P (☐). Dark thick scrawls represent peptide chains. PI: phosphatidylinositol; IPC: inositol phosphoceramide. Types B, C and D were defined from bands obtained using TLC. Image published in Lucas *et al.*, 2016.

S. cerevisiae mature GPI anchors contain either ceramide or diacylglycerol with a C26 fatty acid in the *sn*2 position (Sipos *et al.*, 1997; Fujita *et al.*, 2011). Gup1 was proposed to be the enzyme that adds C26 fatty acids to the *sn*2 position of lyso-phosphatidylinositol (lyso-PI)-containing GPI anchors (Bosson *et al.*, 2006) (Fig. 9).

Accordingly, the *GUP1* deleted mutant, in contrast to the *wt* strain, was shown to accumulate lyso-PI and to contain phosphatidylinositol tails with C16 and C18 fatty acids instead of C26, all found in much smaller amounts compared to *wt* (Fig. 9) (Bosson *et al.*, 2006). The direct involvement of Gup1 as an enzyme in this process is nevertheless controversial. The *wt* GPI-anchor types can still be found in the mutant (Bosson *et al.*, 2006). Concomitantly, it was suggested that Cwh43 was able to catalyse a bypass to the Gup1-dependent step in inositol phosphoceramide (IPC) *tail* maturation (Umemura *et al.*, 2007; Yoko-O *et al.*, 2013), using indifferently PI or Lyso-PI as substrates (Fig. 9). Moreover, the metabolic step associated with Gup1 is preceded by the transformation of PI into lyso-PI by Per1 (Fig. 9) (Fujita *et al.*, 2006). In Δ*gup1* mutants, the lyso-PI harbours different fatty acid chains, suggesting that the Per1-dependent step is affected by the deletion of *GUP1* as well (Bosson *et al.*, 2006). Altogether, it is prudent to restrain from naming Gup1 as a mere GPI remodelase, since this classification does not consider the multiple phenotypes associated with the protein, neither does it consider the localization of Gup1 in the plasma membrane or its association with the mitochondria (Holst *et al.*, 2000). In view of the complexity associated with Gup1 protein so far, the effects observed in GPI anchor synthesis could be indirect. GPI-anchor remodelling, if confirmed to be a direct function of Gup1 as an enzyme, will be one of several functions.

Cell Death

Apoptosis is the most common process of Programmed Cell Death (PCD) in eukaryotes. It is vital for the fast elimination of useless or injured cells, and for the differential development of tissues and organs. The occurrence of PCD in yeast, exhibiting typical morphological and biochemical hallmarks of the process in metazoan, is presently unarguable (Carmona-Gutierrez *et al.*, 2010). As mentioned above, multicellular aggregates of microbial cells, like colonies or biofilms, are spatially organized, and require the specialization of cells differentially localized to ensure supply of nutrients and water to the whole cell ensemble (Palková and Váchová, 2016). PCD occurs in a differentiated way within these aggregates (Váchová and Palková, 2005). The underlying rationale is that the death of older cells at centre of the colony, which have already multiplied extensively, can contribute to the survival of adjacent cells with limited access to nutrients (Váchová and Palková, 2005).

Several types of stimuli, both endogenous and exogenous, are known to cause yeast cells to enter a PCD process. H_2O_2 and acetic acid are major exogenous triggers, commonly used to induce apoptosis in yeast (reviewed by Carmona-Gutierrez *et al.*, 2010). However, several additional agents were reported to induce apoptotic phenotypes, namely, salt stress, ethanol, heavy metals, UV radiation and high temperatures. Endogenous triggers on the other hand, include defects in N-glycosylation, chromatid cohesion, mRNA stability and ubiquitination (reviewed by Carmona-Gutierrez *et al.*, 2010). Moreover, DNA damage (resulting from oxygen metabolism and reactive oxygen species (ROS) generation) and replication failure can stimulate the activation of yeast cell death programmes. PCD may also play a role in yeast ageing, replicative (Laun *et al.*, 2001), or chronological (Herker *et al.*, 2004; Fabrizio and Longo, 2008).

Gup1 is required for several cellular processes that are related to yeast apoptosis, namely the above discussed rafts integrity and stability (Ferreira and Lucas, 2008), lipid metabolism (Oelkers *et al.*, 2000; Ferreira and Lucas, 2008) (including GPI anchor correct remodelling (Bosson *et al.*, 2006)), mitochondrial and vacuole functions (Bonangelino *et al.*, 2002; Reiner *et al.*, 2006), and actin dynamics (Ni and Snyder, 2001; Casamayor and Snyder, 2002). Accordingly, the deletion of *GUP1* induces the hypersensitivity of yeast cells to two known apoptosis inducing conditions (Tulha *et al.*, 2012), acetic acid (Ludovico *et al.*, 2001) and chronological life span (CLS) (Fabrizio and Longo, 2008). In the presence of lethal concentrations of acetic acid, unlike the *wt*, Δ*gup1* undergoes a necrotic-like cell death process (Tulha *et al.*, 2012). This is inferred from the absence of typical apoptotic features, such as: (a) maintenance of the membrane integrity, (b) phosphatidylserine externalization, (c) depolarization of mitochondrial membrane, and (d) chromatin condensation. Moreover, Δ*gup1* acetic acid treated cells display a massive increase of ROS (Tulha *et al.*, 2012). On the other hand, two apparently contradictory works suggest that the *GUP1* deletion decreases (Tulha *et al.*, 2012), or extends (Li *et al.*, 2011) CLS. In this last work, replicative life span (RLS) is also described to increase in the absence of *GUP1*. The main difference between both studies resides in the amino acid availability in the yeast growth medium, which was four times higher in the study by Li *et al.* (2011) than in the one by Tulha *et al.* (2012). Considering the above-mentioned suggestions that Gup1 might be implicated in or regulated by TOR signalling, which is controlled by nitrogen/amino acid abundance

(Loewith and Hall, 2011), the mutant is expected to respond differently in either cultivation condition. This different behaviour in response to amino acids availability could suggest an involvement of Gup1 in the autophagic process.

Autophagy is a tightly regulated mechanism that plays an important role during proper cell growth and cell homeostasis. It involves the recycling of intracellular components of a cell, allowing it to eliminate damaged organelles and to survive when environmental resources are scarce (for a review see Reggiori and Klionsky (2013). The deregulation of autophagy is associated with cell death. Actually, excessive autophagy can lead to an autophagic cell death, while deficient autophagy results in the inability of cells to adapt to unfavourable environmental conditions eventually leading to death (Abeliovich, 2015; Noda and Inagaki, 2015). The putative involvement of Gup1 in this process might explain the decrease of Δ*gup1* mutant CLS in low amino acids conditions above mentioned (Tulha *et al.*, 2012), due to its inability to recycle nutrients through autophagy.

Differentiation and Morphology

The Hh pathway has well-established roles in high eukaryotes development, and the maintenance of differentiation. In yeasts, changes in morphology and differentiation occur at two very distinct levels. Individual cells can differentiate by changing shape, shifting from yeasts to very elongated polarized cells of true or pseudo hyphae, while colonies can display multiple 3D shapes that relate with the ability to invade and adhere (Granek and Magwene, 2010). At this level, *Candida* species, namely *C. albicans*, were more studied than *S. cerevisiae*, mainly due to their pathogenicity. *C. albicans* is a commensal constituent of normal human microflora that acts as an opportunistic pathogen, causing infections such as dental stomatitis, thrush and urinary tract infections, but can also provoke more severe systemic infections, particularly in immunocompromised individuals (Mayer *et al.*, 2013; Eggimann and Pittet, 2014). Among the underlying virulence mechanisms, the most well studied so far is probably the morphological switch from budding yeast to filamentous fungus, which, together with the cell surface expression of adhesins and invasins (reviewed by Mayer *et al.*, 2013), allows colonization of host tissues and their aggressive invasion, eventually overcoming the endothelial barrier (Cheng *et al.*, 2012). The infection-associated yeast fast reproduction promotes the formation of biofilms, not only on human and other

organisms' tissues, but also on inert surfaces from clinical devices (Eggimann and Pittet, 2014; Nobile and Johnson, 2015), representing a major threat for hospitalized patients.

C. albicans has only one *GUP* orthologue, *CaGUP1*, which has been implicated in *C. albicans* virulence (Ferreira *et al.*, 2010). Accordingly, the *CaΔgup1* null mutant was strongly affected in the ability to develop true hyphae, to adhere, invade and form biofilm, and its colonies exhibited aberrant morphology/differentiation patterns (Ferreira *et al.*, 2010). Interestingly, *M. musculus GUP1* – HHATL - cDNA was able to complement the *CaΔgup1* null mutant morphogenic defects associated with colony invasive growth (Ferreira *et al.*, 2010; Lucas *et al.*, 2016). Preliminary data suggests that the hyphae development defects of the mutant can be reverted, although not completely, by *S. cerevisiae GUP1*, as well as the human HHATL and *D. melanogaster* RASP/HHAT - *GUP2* (Ferreira *et al.*, 2010; Armada, 2011). This partial phenotype reversal corresponds to a notorious cell elongation into pseudo instead of true hyphae. Interestingly, the amino acid sequences of *C. albicans* and *M. musculus* Gup proteins share only 20% similarity, whereas *S. cerevisiae* and *C. albicans* proteins share 58% (Lucas *et al.*, 2016). Moreover, as mentioned above, one of the two MBOAT signature amino acid residues differs in yeast Gup proteins and mammalian HHAT(L). It remains to be seen in the future how far the high eukaryotes HHAT(L) ability goes in complementing yeast phenotypes, and if the substitutions in the MBOAT residues play a role.

Another important phenotype of the *CaΔgup1* null mutant is the delayed ability to form biofilm (Ferreira *et al.*, 2010). The formation of biofilms, as the formation of colonies, derives from extensive invasion and colonization of the environment, generating multicellular structures that allow regular feeding of all cells, comparably to a *proto-tissue*. In view of the clinical implications above mentioned, *C. albicans* as other closely related yeasts, were extensively used to study biofilm formation. *S. cerevisiae* can be used to mimic biofilm by promoting its growth onto large communities such as giant colonies or mats (Reynolds and Fink, 2001; Bojsen *et al.*, 2012; Brückner and Mösch, 2012; Faria-Oliveira *et al.*, 2014). Analogously to the tissues from higher eukaryotes, these multicellular aggregates depend on the production and secretion of an extracellular matrix (ECM) (Faria-Oliveira *et al.*, 2014). In *S. cerevisiae*, the ECM is essentially composed of two different polysaccharides (Faria-

Oliveira *et al.*, 2014, 2015a), while *C. albicans* only presents one, the one with highest molecular weight (Faria-Oliveira *et al.*, 2014). Both yeasts ECM harbour a large and abundant proteome (Faria-Oliveira *et al.*, 2014), which in *S. cerevisiae* consists of 684 well-identified proteins belonging to very different functional classes (Faria-Oliveira *et al.*, 2015b). The deletion of *GUP1* in *S. cerevisiae* provokes a profound difference in the composition of this secretome, yielding a sludgy-textured ECM, by loss of the higher molecular weight polysaccharide from *wt* ECM (Faria-Oliveira, 2013). The *ca*Δ*gup1* null mutant was never assessed in that regard, so no information exists as to what might be the consequences for *C. albicans* ECM composition and strength caused by the absence of *Ca*Gup1. The *S. cerevisiae* Δ*gup1* ECM harbours approximately 15% less proteins, 26% of which are not found in the wild-type ECM (Faria-Oliveira *et al.*, 2015a, 2015b). Additionally, DIGE analysis also identified proteins present in both *wt* and mutant ECMs, in statistically different amounts (Faria-Oliveira *et al.*, 2015b). Among the missing proteins, there are (i) key regulators of metabolic pathways, namely Fbp1, Pyc2 and Pdc5/6 from glycolysis/gluconeogenesis/fermentation; (ii) important effectors in cytoskeleton organization, like Tub2, Ent2/3, Rvs161 or VPS; (iii) stress response and secretory pathway proteins like Vma6/7, Vph1 or Ctt1 and Ecm4/38; and (iv) proteins involved in ubiquitination and sumoylation (Faria-Oliveira *et al.*, 2015b). Moreover, the selective presence of α-saccharides remodelling enzymes in the *wt* ECM, such as Glc3 1,4-α-glucan branching enzyme, Mnn2 α-1,2-mannosyltransferase, and Sga1 α-glucoamylase, suggests the presence of α-based polysaccharides. These might be important for ECM texture as inferred from the absence of these enzymes in Δ*gup1* mutant ECM (Faria-Oliveira *et al.*, 2015b). On the other hand, the mutant ECM comprises a great number of proteins involved in cell wall remodelling that were not found in the wild-type ECM, including (i) the homologous GPI-anchored putative mannosidases Dcw1 and Dfg1 required for cell wall biosynthesis, (ii) the Utr2, Kre6 and Krt2 proteins involved in the biosynthesis of β-glucans, (iii) the Pir1 and Pir2 proteins, involved in stabilization of the cell wall, and (iv) the GPI-anchored protease Yps1, required for cell wall growth and maintenance (Faria-Oliveira *et al.*, 2015b). Altogether, these data clearly indicate that Gup1 considerably interferes with the secretion of glycosides and proteins into the yeast ECM.

Gup1/2 Homologues from High Eukaryotes

As discussed above, yeast Gup1 and its close homologue Gup2 share significant sequence homology with proteins belonging to higher eukaryotes, from *Caenorhabditis elegans*, to *Drosophila melanogaster*, *Mus musculus* and other mammals including humans. These proteins, identically to Gup1/2 from yeast, were identified as members of the MBOAT family (Hofmann, 2000). Most members of this family catalyse the transfer of long chain fatty acids to hydroxyl groups of other lipophilic molecules (Cases *et al.*, 1998; Chang *et al.*, 1998; Ståhl *et al.*, 2008), or the formation of wax esters (Yen *et al.*, 2005). They are categorized into three subgroups based on their biochemical reaction (Chang *et al.*, 2011). Mammalian Gup2/HHAT and its fly counterpart RASP, together with Porcupine and Ghrelin *O*-acyltransferase (GOAT), belong to the group involved in the acylation of secreted proteins (Chang *et al.*, 2011; Masumoto *et al.*, 2015). In particular, HHAT is responsible for the N-palmitoylation of the Hh morphogens (Konitsiotis *et al.*, 2015). *M. musculus* and human Gup1/HHATL, as detailed above, have the invariant His residue, necessary for the palmitoylation activity of HHAT, replaced by a Leu residue (Buglino and Resh, 2010; Konitsiotis *et al.*, 2015). HHATL is, therefore, not expected to act as an acyltransferase, although it was found to co-localize with both HHAT and the Hh morphogen in the ER (Buglino and Resh, 2008). It was shown that HHATL interacts directly with Shh, decreasing the efficiency of its palmitoylation (Abe *et al.*, 2008), being therefore proposed to act as a negative regulator of the HHAT-driven N-palmitoylation of Shh and consequently of the Hh pathway. However, the precise mechanism of action has yet to be clarified.

Hedgehog Pathway

In embryonic development and postnatal life, a limited number of signal transduction pathways are repeatedly used both to provide instruction to naïve cells and to control cellular differentiation and tissue formation. The evolutionarily conserved Hh signalling pathway plays an important role in regulating cell growth and differentiation during embryonic development. Hh signalling also remains important throughout adulthood, governing stem cell proliferation, tissue maintenance and regeneration, and wound healing (for a review see Lee *et al.* (2016). Deregulation of this pathway is a cause of

developmental abnormalities and can lead to several diseases (McMahon *et al.*, 2003). The Hh secreted morphogens were originally discovered in genetic screens aimed to provide an understanding of body segmentation in *D. melanogaster* (Nüsslein-Volhard *et al.*, 1980), but members of the Hh family have later been found in all other metazoans. Mammals possess three Hh morphogens involved in long distance cell-cell communication: Desert Hedgehog (Dhh), Indian Hedgehog (Ihh), and the Sonic Hedgehog (Shh) above mentioned (Echelard *et al.*, 1993). The morphogen production, secretion, migration, and transduction processes associated to Hh pathway are conserved among several model organisms (Guerrero and Chiang, 2007; Gallet, 2011).

Morphogen production and processing

Hh proteins are synthesized as precursor proteins (around 45 kDa) that contain a signal sequence for the trafficking into the ER (Lee *et al.*, 1992). The signal sequence is then cleaved, and the C-terminal half of the remaining Hh protein undergoes a self-catalysed, autoproteolysis reaction, that cleaves the peptide in two parts (Lee *et al.*, 1994). This self-splicing process is promoted by the addition of a cholesterol molecule to the C-terminal domain (Porter *et al.*, 1996). The N-terminal domain is subsequently palmitoylated by HHAT (Pepinsky *et al.*, 1998; Chamoun *et al.*, 2001; Lee *et al.*, 2001; Buglino and Resh, 2008) (Fig. 10), which uses palmitoyl-CoA as preferential substrate to transfer the palmitate to the N-terminal domain via an amide linkage (Buglino and Resh, 2008). As mentioned before, the negative regulation of this N-terminal Hh palmitoylation is performed by HHATL (Abe *et al.*, 2008). The so-obtained 19 kDa protein, that contains the C-terminal cholesterol and the N-terminal palmitate (Fig. 10), is the mature form of the Hh protein and the prevalent form secreted into the extracellular space (Taipale *et al.*, 2000).

Proper morphogen signalling is achieved by a specific gradient of Hh morphogens concentration, from the cells that produce it to those destined to bind them (Gritli-Linde *et al.*, 2001; Stamataki *et al.*, 2005; Fuccillo *et al.*, 2006). Hh post-translational lipophilic modifications have a key role in maintaining the protein concentration gradient, influencing the long-range signalling (Callejo *et al.*, 2008). Accordingly, palmitoylation at the N-terminal domain of Hh proteins is necessary for the proper Hh signalling at both long and short range (Lee *et al.*, 2001). It was shown *in vitro* that palmitoylated forms of Shh are 40–160-fold more active compared to unmodified Shh

(Pepinsky *et al.*, 1998). Moreover, HHAT loss of function by a single G287V missense mutation resulted in serious sex development defects in humans (Callier *et al.*, 2014). Therefore, the proper functionality of HHAT is essential to maintain the correct Hh signalling function, which agrees with the importance of the N-terminal palmitoylation of Hh for signalling.

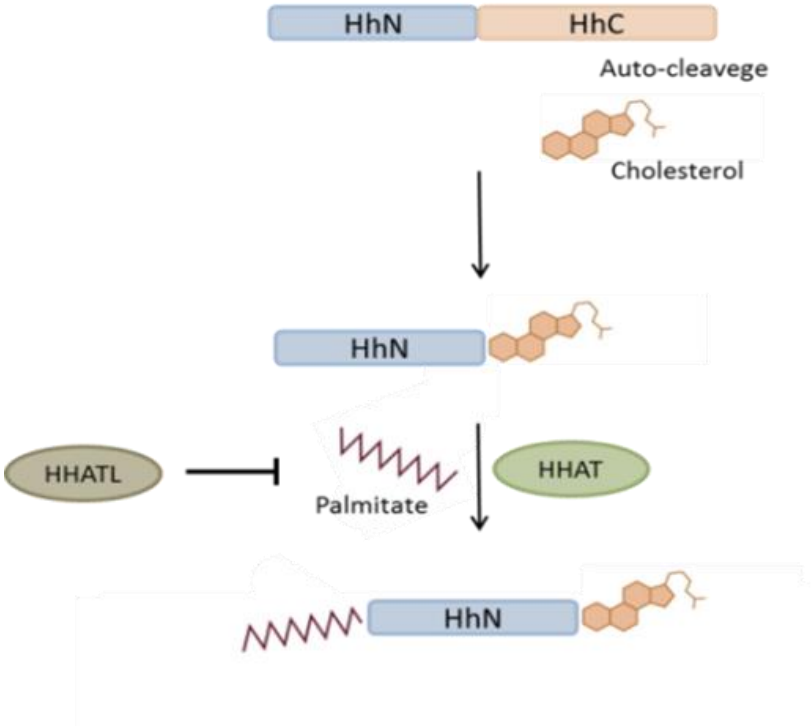

Figure 10 - Simplified scheme of a Hh morphogen maturation processes. Hh is synthesized as a precursor, which undergoes self-cleavage to liberate a 19 kDa N-terminal signaling fragment (HhN) (blue segment) and a C-terminal autocatalytic fragment (HhC) (orange segment). The self-cleavage is promoted by the addiction of a cholesterol molecule to the C-terminal of the HhN. The N-terminus of HhN is also modified via the addition of a palmitate molecule by HHAT. The lipid-modified Hh protein is then released from the Hh secreting cell into the extracellular space.

Morphogen secretion and migration

The hydrophobic nature of the lipid modifications does not allow the Hh morphogen to diffuse freely through the plasma membrane, promoting it association with sterol-rich membrane microdomains (Rietveld *et al.*, 1999). Therefore, several mechanisms were suggested for the secretion and diffusion of the Hh morphogens (Briscoe and Thérond, 2013) (Fig. 11). The lipid modified Hh monomers could be released through the action of the multipass transmembrane protein Dispatched (Disp) (Burke *et al.*, 1999; Tukachinsky *et al.*, 2012). Monomeric Hh can also self-associate to form large soluble multimeric complexes that are released from the membrane (Zeng *et al.*, 2001; Goetz *et*

al., 2006). The formation of these multimeric complexes depends on the dual lipid Hh modification, since Hh proteins lacking cholesterol or palmitate cannot form multimers, and this results in defects in the long-range spread and signalling (Lewis *et al.*, 2001; Zeng *et al.*, 2001; Gallet *et al.*, 2003; Callejo *et al.*, 2006).

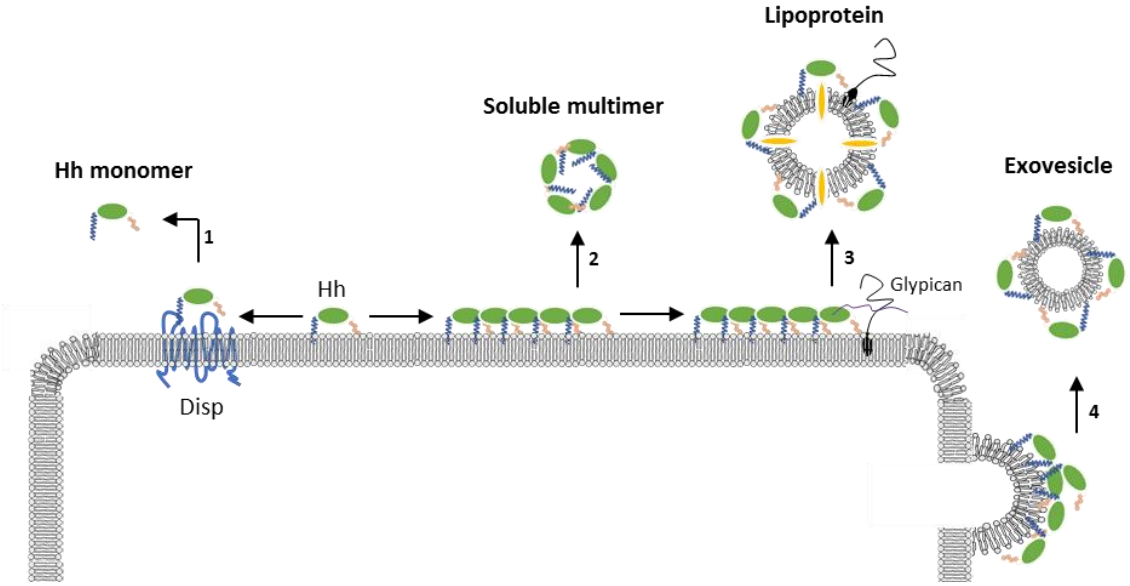

Figure 11 - Key mechanism for Hh morphogen secretion. Once at the outer surface of the plasma membrane, dually lipid-modified HH-N is associated with the lipid bilayer as a monomer until it is released by one of four key mechanisms: 1) Hh monomers can be released through the action of transmembrane protein Disp; 2) Monomeric Hh can self-associate to form large soluble multimers that are released from the membrane; 3) Hh oligomers can assemble into lipoprotein particles; and 4) Hh can also be released at the surface of exovesicles.

Another mechanism is the formation of diffusible lipoprotein particles (Panáková *et al.*, 2005; Steinhauer and Treisman, 2009). The Hh oligomers can interact with the heparan sulphate chains of glypicans (a type of heparin sulphate that attach to the outer surface of the plasma membrane by a GPI anchor). These enable the Hh oligomers to recruit lipophorin apolipoproteins and assemble into lipoprotein particles (Panáková *et al.*, 2005; Eugster *et al.*, 2007; Gallet *et al.*, 2008). The glypican GPI anchor might be cleaved from the cell surface by a phospholipase C-like protein, allowing the release of Hh in large soluble lipoprotein particles (Eugster *et al.*, 2007). Glypicans are also important for Hh to spread from cell to cell through the ECM, and promote long-range signalling (Han *et al.*, 2004; Koziel *et al.*, 2004).

Alternatively, Hh could be released by extracellular vesicular particles, through an exosome-mediated pathway (Liégeois *et al.*, 2006; Vyas *et al.*, 2014). Exosomes derive from the endocytic compartment where vesicles are packed (Raposo and Stoorvogel, 2013). During the process of exosome assembly different proteins are assorted, originating different secreted vesicles with different signalling roles. Hh morphogen, as other functional signalling molecules, was previously detected on extracellular vesicles such as exosomes (Liégeois *et al.*, 2006; Vyas *et al.*, 2014; Vyas and Dhawan, 2017).

Hh signalling transduction

In Hh responsive cells, the signalling is initiated when Hh binds to Patched (Ptch) receptors, a transmembrane protein that constitutively represses Hh signalling, present on the cell surface (Fig. 12). This binding leads to the internalization and degradation of Ptch (Marigo *et al.*, 1996; Stone *et al.*, 1996). The Hh binding to Ptch and its subsequent removal from the plasma membrane relieves the repression of another protein, Smoothened (Smo), allowing it to move from an intracellular compartment to the cell surface (Stone *et al.*, 1996; Taipale *et al.*, 2002). This kind of derepression results in the activation of a cytoplasmic transduction cascade to the nucleus: activated Smo promotes the disassembly of the protein complex formed by Sufu and a Gli transcription factor, which is therefore activated, migrating to the nucleus and activating the expression of target genes (Kogerman *et al.*, 1999; Sasaki *et al.*, 1999; Cheng and Bishop, 2002; Paces-Fessy *et al.*, 2004). In the absence of Hh, the pathway is turned off by Ptch, which inhibits Smo, resulting in Gli sequestration in the cytoplasm. Cytoplasmic sequestration of the Gli transcription factors by Sufu facilitates the processing of Gli proteins generating a repressed form (Gli-R), therefore inhibiting the following downstream Hh signalling events (Taipale *et al.*, 2002; Eaton, 2008; Wilson and Chuang, 2010). Thus, the relative abundance of activated and repressed forms of Gli ultimately regulates the transcription of Hh target genes.

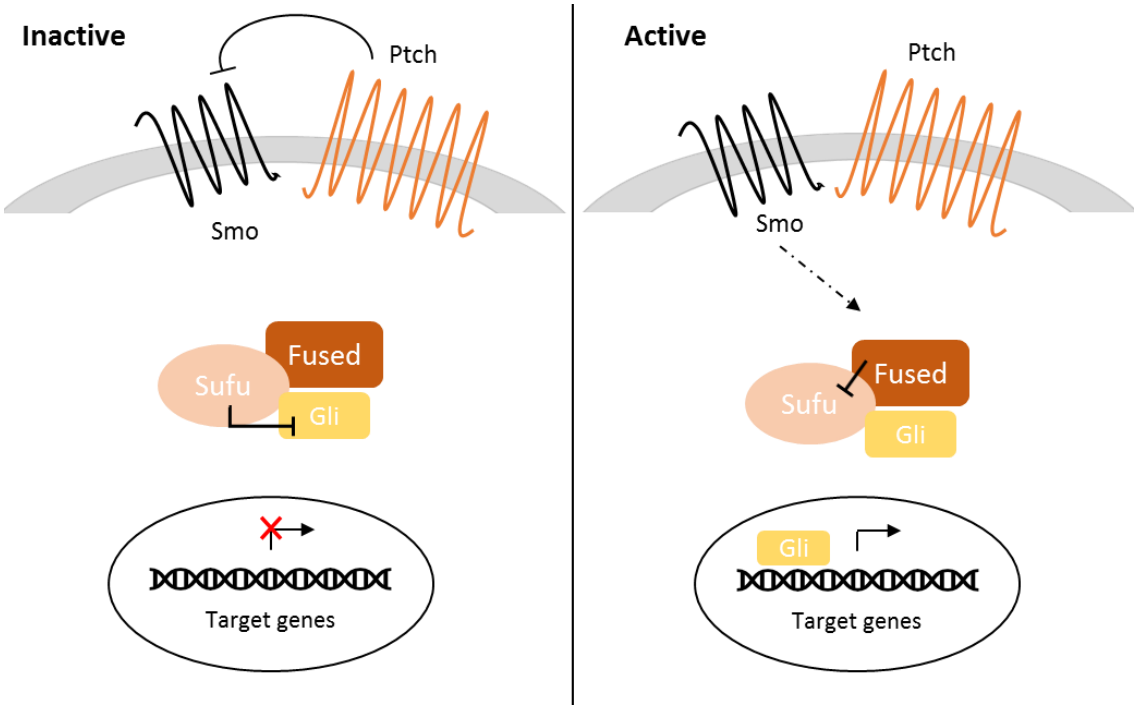

Figure 12 - Simplified scheme of the Hh signaling pathway. In the absence of ligand, the Hh signaling pathway is inactive (left). The transmembrane protein receptor Ptch inhibits the activity of another transmembrane protein Smo. The transcription factor Gli, a downstream component of Hh signaling, is prevented from entering the nucleus through interactions with the cytoplasmic effectors Fused and Sufu. Consequently, transcriptional activation of Hh target genes is repressed. Activation of the pathway is initiated through binding of Hh to Ptch (right). Ligand binding results in de-repression of Smo, thus activating a cascade that leads to the translocation of the active form of the transcription factor Gli to the nucleus. Nuclear Gli activates target gene expression.

In addition to the classical signalling, there are also non-canonical pathways related to Hh signalling. These correspond to the activation of signalling from Ptc1/Smo in a Gli-independent manner, or the activation of Gli transcription factors by Smo-independent mechanisms. This latter one is better studied and could result in the increase of Hh signalling even in the absence of the Hh ligand. A non-canonical activation of Gli1 was described in association with oesophageal adenocarcinoma (Wang *et al.*, 2012). In that case, Gli1 is controlled by a TNFα-derived TORC1/S6K1 pathway. TORC1 promotes the phosphorylation of Gli1 (at S^{384}) by S6K1, freeing it from SuFu and facilitating its migration to the nucleus. Additionally, TORC2 promotes the stabilization of Gli1 by keeping it phosphorylated (at S^{230}) through the action of AKT (Stecca *et al.*, 2007; Wang *et al.*, 2012). Moreover, Gli transcription factors were shown to be positively regulated by K-Ras, PI3K-AKT, TGF-β, and PKC-α (Seto *et al.*,

2009; Rajurkar *et al.*, 2012; Zhou *et al.*, 2016). K-Ras pathway, in particular, seems to be capable of activating Gli1 independently of the Hh pathway, as knockdown of Sufu does not affect K-Ras-induced Gli1 activation (Rajurkar *et al.*, 2012). Additionally, the Gli proteins were shown to be negatively regulated by p53, PKA, and PKC-δ (Stecca and Altaba, 2009; Makinodan and Marneros, 2012; Yoon *et al.*, 2015).

Hh Signalling and HHAT(L) Associated Pathologies

Deregulation of Hh signalling is associated with a variety of human diseases ranging from developmental disorders to certain forms of cancer. In general, Hh signalling malfunction can cause severe and often life-threatening developmental disorders such as holoprosencephaly, Pallister–Hall syndrome (polydactyly), craniofacial defects and skeletal malformations (Muenke and Beachy, 2000; McMahon *et al.*, 2003; Hill *et al.*, 2007). Moreover, it has been estimated that 25% of all human tumours require aberrant Hh signalling activation to maintain tumour cell viability (Andrade and Einsle, 2007). In fact, corrupted Hh signalling, underlies many types of cancer, including small and non-small cell lung cancer, squamous cell and basal cell carcinomas and myeloid leukemias (Karhadkar *et al.*, 2004; Feldmann *et al.*, 2007; Jacob and Lum, 2007; Yauch *et al.*, 2008; Tian *et al.*, 2009). The importance of Hh signal palmitoylation on this regard has recently been demonstrated. The knockdown of HHAT caused *in vitro* reduced proliferation and invasiveness of human pancreatic ductal adenocarcinoma (Konitsiotis *et al.*, 2014). Therefore, it is conceivable that blocking the Hh pathway using HHAT inhibitors may block tumour progression (Matevossian and Resh, 2015; Petrova *et al.*, 2015). Moreover, HHATL transfected cells presented lower capability of cell proliferation, invasion and tumorigenicity, supporting the notion that the protein might function as a tumour suppressor (Zhang *et al.*, 2005). This function is compatible with the negative regulation of HHAT-mediated Hh palmitoylation described for HHATL (Abe *et al.*, 2008). The c3orf3 gene (one of the numerous *GUP1*/HHATL aliases (Table 1) is expressed in very few tissues and in different amounts (heart > skeletal muscle > brain) (Soejima *et al.*, 2001; Zhang *et al.*, 2005; The Human Protein Atlas: http://www.proteinatlas.org/). The reason why this gene is only expressed in few tissues is intriguing. Other works reported the expression of KIAA1173 (another *GUP1*/HHATL aliases, Table 1) in normal skin (Zhang *et al.*, 2010) and nasopharyngeal mucosa (Zhang *et al.*, 2005), and its extreme down-regulation in cells

of skin squamous cell carcinoma (Zhang *et al.*, 2010). Still, the KIAA1173 protein is N-172 amino acids shorter than the HHATL cardiac isoform c3orf3 above-mentioned (Soejima *et al.*, 2001), suggesting that the HHATL ORF might undergo extensive RNA or protein splicing and perform different roles in different tissues.

The HHAT gene has also been associated with type 2 diabetes (Berisha *et al.*, 2011). Its changed expression was found in patients after bariatric surgery (Berisha *et al.*, 2011), further suggesting a correlation with glucose homeostasis and therefore obesity. *Diabetes mellitus* and obesity are closely related (Al-Goblan *et al.*, 2014), through deficient glycerol consumption by glycerol kinase (Rahib *et al.*, 2007), and deficient tissue regulation of glycerol transport and retention by aquaglyceroporins (Maeda *et al.*, 2008). This recalls the original observation by Holst *et al.* (2000) that in yeasts Gup1 is implicated in the regulation of glycerol transport, as well as its consumption and production for osmoregulation purposes (Holst *et al.*, 2000; Neves *et al.*, 2004b; Ferreira *et al.*, 2006). Furthermore, the implication of HHAT in diabetes and obesity through glycerol appears to connect nutrient regulation to Hedgehog-driven morphogenesis. All taken, this appears a signalling entanglement between the CWI/PKC, TORC2/YPK, TORC1 and HOG pathways, in which Gup proteins could perform multiple roles as a kind of information hub.

Scope of the thesis

The *S. cerevisiae* Gup1 is associated with a high number of diverse biological functions. Besides the original glycerol transport-related phenotypes, the deletion of *GUP1* is also associated with crucial yeast cell biological processes such as wall and membrane integrity, structure and composition, cytoskeleton/polarity and bud-site selection, secretory/endocytic pathway functionality, and vacuole morphology. Phenotypes underlying death, morphogenesis and differentiation are also associated with *GUP1* deletion. Moreover, in *C. albicans*, the only Gup protein, *Ca*Gup1, was implicated with virulence, interfering with the capacity to develop hyphae, to adhere, to invade, and to form a biofilm.

The mammalian Gup2 and Gup1 homologues HHAT and HHATL, are key proteins in the morphogenic Hh signalling pathway, that operates in high Eukaryotes, during embryogenesis and wound healing. Gup2/HHAT palmitoylates the Hh morphogens prior to their secretion, while Gup1/HHATL negatively regulates this function, and consequently Hh pathway. Paracrine signalling, like the one promoted by the Hh pathway, has never been described in yeasts or other microbes communities. Still, unicellular organism like yeast can form large aggregates of cells like biofilms or colonies, that display a tissue-like behaviour, and encompass, according to present state of the art, a cell-to-cell communication system, and population density sensing, operating through the secretion of small molecules.

The roles that Gup proteins homologues perform in mammalian models, combined with the extensive series of yeast cellular processes in which this protein interferes, inferred from the extensive phenotypes associated with *GUP1* deletion, and the multiple sub-cellular localizations of Gup1, suggest the protein has multiple roles and therefore should have also multiple partners. Several proteins have been suggested to interact physically with Gup1, as result of HTP screenings. One exception is the ammonium transceptor Mep2 that was addressed in detail though a function for the partnership was not found. Therefore, the main goal of the present thesis was to identify the intracellular partners of Gup1, as a first step to unveil the molecular function of this protein.

From the many phenotypes described and studied in detail in association with *GUP1* deletion, some are in line with the processes controlled by Hh pathway in higher Eukaryotes. These include the capacity to develop hyphae, to adhere, to invade, and to form a biofilm above mentioned. These were though previously assessed in *C. albicans* not in *S. cerevisiae*. In this thesis, simple assays were performed to assess whether those basic processes were being affected by the deletion of *GUP1*, a partner, or by their association. This was done together with the assessment of the main processes previously described in association with *GUP1* deletion, *i.e.* the cell wall and membrane integrity, and stress response phenotypes. By applying this strategy for each of the proteins identified as a physical partner of Gup1, the ones found within the work of this thesis, and the previously characterized Mep2, this work ambitioned to identify the cellular niche of each Gup1-Partner interaction. The thesis will therefore identify the intracellular physical partners of Gup1 and the cellular processes in which their interaction may impact.

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CHAPTER 2

Identification of novel Gup1 physical partners

Abstract

Saccharomyces cerevisiae membrane-bound *O*-acyltransferase Gup1 is the yeast orthologue of mammalian HHATL, the negative regulator of Hedgehog morphogen secretion. The deletion of *GUP1* in *S. cerevisiae* has been associated with many cellular processes, namely, plasma membrane and cell wall structure, lipid metabolism, trafficking, cytoskeleton organization and budding pattern, extracellular matrix composition. In *Candida albicans*, the annulment of the single *CaGUP1* gene causes defects in morphological switch, biofilm formation, virulence and antifungal resistance. Several studies, mostly whole genome screenings, predicted many Gup1 putative physical partners. These proteins, as Gup1, have diverse cellular localizations, which point to the possible existence of multiple partners for Gup1, according to its intracellular localizations.

The present work aimed at identifying the intracellular partners of Gup1p in *S. cerevisiae*, as a first step to identify the molecular role/s of this protein. Two approaches were used: (1) to express *Sc*Gup1 in *Escherichia coli* and purify it, in order to obtain a suitable amount of protein to proceed to affinity chromatography, and (2) to co-immunoprecipitate Gup1 and its partners in native conditions using Gup1 as bait. In this particular case, all attempts to express Gup1 in *E. coli* were unsuccessful. On the other hand, using the co-immunoprecipitation technique, two novel Gup1 physical interactions were found: the yeast mitochondrial VDAC (Voltage-Dependent Anion Channel) Por1, and the eisosome major component Pil1. These proteins localize in the outer mitochondrial membrane and the plasma membrane, respectively. This reinforces the notion that Gup1 could interact with different proteins on different localizations as a requisite for multiple functions, or the node-type control of multiple pathways, as predicted by the multiple phenotypes associated with this protein deletion.

Introduction

Saccharomyces cerevisiae GUP1 is involved in a wide range of cellular processes including cell wall and membrane composition, rafts assembly, lipid metabolism, GPI-anchor remodelling, cytoskeleton polarization, trafficking, vacuole morphology, telomere length, life span and cell death, and ECM composition (Oelkers *et al.*, 2000; Ni and Snyder, 2001; Bonangelino *et al.*, 2002; Askree *et al.*, 2004; Bosson *et al.*, 2006; Ferreira *et al.*, 2006, 2010; Reiner *et al.*, 2006; Ferreira and Lucas, 2008; Tulha *et al.*, 2012; Faria-Oliveira *et al.*, 2015a, 2015b). Moreover, this gene has attracted a great deal of attention because of its higher eukaryotes homologues, the HHATL proteins, which act as negative regulators of the N-palmitoylation of the Hedgehog (Hh) secreted signal (Abe *et al.*, 2008). Hh pathway plays an important role in the development processes regulating morphogenesis, differentiation and patterning during embryogenesis, including proliferation and cell fate (Varjosalo and Taipale, 2007; Lee *et al.*, 2016).

GUP genes are ubiquitous to all eukaryotes which genome has been sequenced so far. But Hh pathway has not been ascertained to yeasts, in spite of the recognizable cellular and colony morphology shifts (Soll *et al.*, 1994; Granek and Magwene, 2010), and consequent multicellular tissue-like biology of biofilms (Palková and Váchová, 2016). In particular, the wide variety of phenotypes observed in the Δ*gup1* mutants (Ni and Snyder, 2001; Ferreira *et al.*, 2010) aligns with the putative existence of some Hh-like pathway able to coordinate proliferation/survival together with morphology. It is therefore urgent to identify the Gup1 direct partners as a first step towards the confirmation or disclosure of the existence of such a pathway in yeasts.

Whole genome screenings revealed the putative Gup1 and Gup2 interactome (SGD database) (Table 1). Besides, one single more focused study identified the ammonium permease Mep2 as Gup1 partner (Van Zeebroeck *et al.*, 2011). All those proteins, as expected from their roles, are found in different parts of the cell: cytoplasm, nucleus, vacuole, mitochondria and plasma membrane. Gup1 was reported to locate primarily in the plasma membrane and ER (Hölst *et al.*, 2000; Bleve *et al.*, 2005), but also to the mitochondria (Hölst *et al.*, 2000). These Gup1 multiple localizations allow to consider the possibility that this protein might interact with different partners in its different localizations, acting as a coordination node, connecting specific pathways, analogously to what is known for major effectors like Cdc42 (Rincón *et al.*, 2014).

Table 1 - Gup1 and Gup2 ascertained and predicted physical partners

	Partner	Assigned Function	Ref.
Gup1	Fet3	Iron-O_2-oxidoreductase; multicopper oxidase that oxidizes ferrous (Fe^{2+}) to ferric iron (Fe^{3+}) for subsequent cellular uptake by transmembrane permease Ftr1.	(Miller *et al.*, 2005)
	Frk1	Protein kinase of unknown cellular role; interacts with rRNA transcription and ribosome biogenesis factors, and the long chain fatty acyl-CoA synthetase Faa3p.	(Ptacek *et al.*, 2005)
	Hek2	RNA-binding protein involved in asymmetric localization of ASH1 mRNA; represses translation of ASH1 mRNA; regulates telomere position effect and length.	(Hasegawa *et al.*, 2008)
	Mep2	NH4+ permease; regulation of pseudo hyphal growth; expression regulated by nitrogen catabolite repression (NCR).	(Van Zeebroeck *et al.*, 2011)
	Msc7	Cytoplasmic protein of unknown function.	(Schlecht *et al.*, 2012)
	Nab2	Nuclear poly(A)-binding protein; required for nuclear mRNA export and tail length control.	(Batisse *et al.*, 2009)
	Sat4	Ser/Thr protein kinase involved in salt tolerance; functions in regulation of Trk1-Trk2 potassium transporter.	(Ptacek *et al.*, 2005)
	Vtc4	Vacuolar membrane polyP polymerase; subunit of the vacuolar transporter chaperone (VTC) complex; regulates membrane trafficking; role in non-autophagic vacuolar fusion.	(Miller *et al.*, 2005)
	YHL042W	Putative protein of unknown function.	(Miller *et al.*, 2005)
Gup2	Aqy1	Spore-specific aquaporin.	(Miller *et al.*, 2005)
	Aus1	Plasma membrane sterol transporter of the ATP-binding cassette family; required, along with Pdr11, for uptake of exogenous sterols and their incorporation into the plasma membrane.	(Snider *et al.*, 2013)
	Hsp30	Negative regulator of the H(+)-ATPase Pma1; stress-responsive; induced by heat shock, ethanol, weak organic acid, glucose limitation, and entry into stationary phase.	(Miller *et al.*, 2005)
	Ifa38	Microsomal beta-keto-reductase; mutants exhibit reduced VLCFA synthesis, accumulate high levels of dihydrosphingosine, phytosphingosine and medium-chain ceramides.	(Miller *et al.*, 2005)
	Nam7	ATP-dependent RNA helicase.	(Johansson *et al.*, 2007)
	Pdr10	ATP-binding cassette transporter; multidrug transporter involved in the pleiotropic drug resistance network; regulated by Pdr1p and Pdr3p.	(Snider *et al.*, 2013)
	Pho88	Probable membrane protein involved in phosphate transport; role in the maturation of secretory proteins.	(Miller *et al.*, 2005)
	Sss1	Subunit of the Sec61 translocation complex (Sec61-Sss1-Sbh1); this complex form a channel for passage of secretory proteins through the ER membrane.	(Miller *et al.*, 2005)
	Ste2	Receptor for α-factor pheromone; interacts with both pheromone and a heterotrimeric G-protein to initiate the signalling response that leads to mating.	(Miller *et al.*, 2005)

The present work endeavoured different approaches to enable the identification of Gup1 physical partners through (1) heterologous expression of Gup in *Escherichia coli* and purification by affinity chromatography, and (2) co-immunoprecipitation (Co-IP). The first method allows to produce and purify Gup1 in a column matrix that can be further used to catch the physical partners of Gup1. This was attempted using two different strategies, but did not succeed. The second method permits the identification of *in vivo* interactions and allowed the finding of two novel Gup1 physical partners.

Materials and Methods

Strains and growth conditions

The *S. cerevisiae* and *E. coli* strains used in this study are listed in Table 2. Yeast cell cultures were grown in either YPD (1% yeast extract, 2% peptone, 2% glucose and 2% agar for solid medium) or YNB medium (0.175% YNB without amino acids and nitrogen source (Difco), 2% glucose, 0.5% $(NH_4)_2SO_4$ and 2% agar for solid medium), supplemented to meet auxotrophic requirements. Growth in liquid media was done at 30 ºC and 200 rpm orbital shaking. Transformed yeast cells were kept in YNB glucose-selective medium and untransformed strains were kept in YPD. Bacteria were grown in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl and 2% agar for solid medium, pH 7.2). The transformed *E. coli* cells were kept in LB solid medium supplemented with ampicillin (100 mg/mL) or kanamycin (50 μg/mL). Growth in liquid media was done at 37 °C and 200 rpm orbital shaking. Induction of *GUP1* expression in bacteria harbouring the correspondent construction in pET-25b(+) and pET-29b(+) induction plasmid was tested at 18, 30 and 37°C, in the presence of 0.1, 0.5, 1 and 2 mM of β-D-1-thiogalactopyranoside (IPTG) during 2, 4, 6, 8, 16 and 22 hours. Lactose (2%)/ 22 h incubation was also used to promote induction.

Table 2 - Yeast and bacterial strains used in this study.

Strain	Genotype	Source
S. cerevisiae W303-1A wt	*MATa; leu2-3,112; trp1-1; can1-100; ura3-1; ade2-1; his3-11,15*	(Thomas and Rothstein, 1989)
S. cerevisiae W303-1A Δ*gup1*	*MATa; leu2-3,112; trp1-1; can1-100; ura3-1; ade2-1; YGL084c::HIS5*	(Hölst *et al.*, 2000)
S. cerevisiae W303-1A Δ*gup1* pYES2 Ø	*MATa; leu2-3,112; trp1-1; can1-100; ade2-1; YGL084c::HIS5*	This study
S. cerevisiae W303-1A Δ*gup1* pYES2-*GFP*	*MATa; leu2-3,112; trp1-1; can1-100; ade2-1; YGL084c::HIS5*	This study
S. cerevisiae W303-1A Δ*gup1* pYES2-*GUP1-GFP*	*MATa; leu2-3,112; trp1-1; can1-100; ade2-1; YGL084c::HIS5*	This study
E. coli XL1 Blue	*endA1 gyrA96(nalR) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB+ lacIq Δ(lacZ)M15] hsdR17(rK- mK+)*	Stratagene
E. coli XL1 Blue pET-25b(+)Ø	*E. coli* XL1 Blue transformed with pET-25b(+)Ø	This study
E. coli XL1 Blue pET-25b(+)-*GUP1*-6xHis	*E. coli* XL1 Blue transformed with pET-25b(+)-*GUP1*-6xHis	This study
E. coli XL1 Blue pET-29a(+)Ø	*E. coli* XL1 Blue transformed with pET-29a(+)Ø	This study
E. coli XL1 Blue pET-29a(+)-*GUP1*-6xHis	*E. coli* XL1 Blue transformed with pET-29a(+)-*GUP1*-6xHis	This study
E. coli BL21(DE3)-CodonPlus-RIL	*F− OmpT hsdSB(rB− mB−) gal dcm (DE3) endA Hte [argU proL Camr] [argU ileY leuW Strep/Specr]*	Stratagene
E. coli BL21(DE3)-CodonPlus-RIL pET-25b(+)Ø	*E. coli* BL21(DE3)-CodonPlus-RIL transformed with pET-25b(+)Ø	This study
E. coli BL21(DE3)-CodonPlus-RIL pET-25b(+)-*GUP1*-6xHis	*E. coli* BL21(DE3)-CodonPlus-RIL transformed with pET-25b(+)-*GUP1*-6xHis	This study
E. coli BL21(DE3)-CodonPlus-RIL pET-29a(+)Ø	*E. coli* BL21(DE3)-CodonPlus-RIL transformed with pET-29a(+)Ø	This study
E. coli BL21(DE3)-CodonPlus-RIL pET-29a(+)-*GUP1*-6xHis	*E. coli* BL21(DE3)-CodonPlus-RIL transformed with pET-29a(+)-*GUP1*-6xHis	This study

Plasmid construction and DNA manipulation

The plasmids used in this work are listed in Table 3.

Table 3 - List of plasmids used in the present study and their origin.

Name	**Source**
pET-25b(+)Ø	M. Casal, CBMA, Universidade do Minho
pET-25b(+)-*GUP1*-6xHis	This study
pET-29a(+)Ø	M. Casal, CBMA, Universidade do Minho
pET-29a(+)-*GUP1*-6xHis	This study
pYES2Ø	Addgene
pYES2–*GFP*	This study
pYES2–*GUP1-GFP*	(Bleve *et al.*, 2005)

GUP1 was amplified by PCR from *S. cerevisiae* W303-1A using the primers A and B (Table 4). These primers were built as to eliminate the stop codon and to include the restriction sites HindIII and XhoI at the 5' and 3' ends, respectively. The resulting PCR product was verified by DNA electrophoresis, and extracted and purified from the gel with DNA Clean & Concentrator™ kit, (ZymoResearch) following the manufacture's recommendations. The purified amplicon was digested with HindIII and XhoI enzymes (NZYTech) and inserted into the pET-25b(+) and pET-29b(+) plasmids (kindly provided by M. Casal, CBMA, Universidade do Minho), through the action of a T4 ligase (Roche). These plasmids are built so to insert in the clones amplicon a His6x tag. These constructions were amplified in *E. coli* XL1Blue using standard procedures (Ausubel *et al.*, 1996), extracted using a Sigma GenElute^{TM} Plasmid extraction kit, and verified by digestion with restriction endonucleases and sequencing. The resulting plasmids pET-25b(+)-*GUP1*-6xHis and pET-29b(+)-*GUP1*-6xHis, as well as the empty vectors were then transformed in *E.coli* XL1 Blue and in *E.coli* BL21(DE3)-CodonPlus-RIL. To build the pYES-*GFP* plasmid, the GFP fragment was amplified from the pYES2-*GUP1-GFP* (kindly provided by G. Bleve, ISPA, Unità di Lecce) with the primers C and D (Table 4) that contains the BamHI and EcoRI restriction sites, respectively. The fragment was cloned in the pYES2 vector following the same strategy described above. The plasmids pYES2-*GFP*, pYES2-*GUP1-GFP* and the empty vector were then used to transform the W303-1A Δ*gup1*. All transformations were confirmed by colony PCR.

Table 4 - List and sequence of the primers used in this work.

Name	Sequence
A - Fw HindIII-*GUP1*	5' AAATTTAAGCTTATGTCGCTGATCAGCATCCTG 3'
B - Rv *GUP1*-XhoI	5' AAATTTCTCGAGGCATTTTAGGTAAATTCCGTG 3'
C - Fw BamHI-*GFP*	5' CACGGATCCTCTAAAGGTGAAGAATTATTC 3'
D - Rv *GFP*-EcoRI	5' GCGGAATTCTAATTTGTACAATTCATCCAT 3'

Co-Immunoprecipitation methodology (Co-IP)

S. cerevisiae W303-1A Δ*gup1* was transformed with the pYES2-*GUP1-GFP* plasmid, the pYES2-*GFP*, or with the pYES2Ø empty plasmid using general yeast plasmid transformation protocol (Ito *et al.*, 1983). Expression was induced incubating for 6 h in YNB with 2 % galactose. For the Co-IP, 250 mL of Δ*gup1* strain expressing Gup1p-*GFP* were collected by centrifugation at 5000 rpm for 5 min at 4°C. The pellet was resuspended in 1 vol. of lysis buffer (50 mM Tris-HCl, pH7.5; 100 mM NaCl; 2 mM EDTA) containing proteases inhibitors (0.4 µl/ml aprotinin; 1 µg/ml leupeptin; 1 µg/ml pepstatin; 1 mM PMSF). Cell lysis was accomplished with 4 cycles of 1 min vortexing the cell suspension with 1 vol. of 0.5 mm ø glass beads, intercalated with 1 min cooling on ice. IP buffer 10x (500 mM Tris-HCl, pH7.5; 1 M NaCl, 20 mM EDTA; 10% NP-40) was added to the collected cell lysate to a final concentration of 1x, and incubated 4 h at 4ºC with constant gentle mixing. Antibody-conjugated beads (anti-GFP (Roche) - Dynabeads® (Invitrogen)), prepared following manufacturer's instructions, were mixed with the cell lysates and incubated overnight at 4ºC. The beads, with the captured proteins, were subsequently washed 3 times with IP buffer 1x, and once with PBS 1x (137 mM NaCl; 2.7 mM KCl; 100 mM Na_2HPO_4; 2 mM KH_2PO_4; pH 7.4) using a magnetic stand, resuspended in 50 µL elution buffer (Laemmli buffer) and boiled for 30 min at 70ºC. The resulting co-immunoprecipitated samples were separated by 12% SDS–PAGE, and the proteins were visualized by colloidal Coomassie Blue or silver staining as previously described (Yan *et al.*, 2000; Dyballa and Metzger, 2009). Bands of interest were excised from the gel and identified by mass spectrometry (Peptide mass Fingerprinting and MALDI-TOF) (purchased from the Unidad de Proteómica, Parque Científico de Madrid, UCM).

Protein precipitation, SDS-PAGE and Western Blotting

Yeast total protein extracts were prepared as previously described (Sivaraman *et al.*, 1997): proteins were precipitated with 3M TCA (trichloroacetic acid), washed with acetone, and solubilized in 2x Laemmli buffer (Laemmli, 1970). Samples were separated by 12% SDS–PAGE, transferred to a PVDF membrane (Roche), blocked with 5% fat-milk in PBS containing 0.05% Tween 20, and incubated with the primary antibody (anti-His 1:2000 (Sigma); anti-GFP 1:2500 (Roche)). Membranes were then incubated with secondary antibodies against mouse IgG (1:10000 (Sigma)), and revealed by chemiluminescence (ECL + Amersham) according to manufacturer's instructions.

Fluorescence microscopy

Fluorescence studies were performed in a Leica Microsystems DM-5000B epifluorescence microscope with the appropriate filter settings using a 100x oil-immersion objective. Images were acquired through a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software

Results and Discussion

Heterologous expression of *GUP1* in *E. coli*

Heterologous expression of *GUP1-6xHIS* in *E. coli* was tempted as a first step to produce and isolate the Gup1 protein. The *GUP1* gene was cloned into two different vectors, the pET-25-a(+) and the pET-29-a(+), both carrying a C-terminal His-tag, and used to transform *E. coli* XL1Blue and BL21 (D3) strains. Several induction conditions were tried, varying on medium, time and temperature (Sarramegna *et al.*, 2003; Wang *et al.*, 2003; Bird *et al.*, 2004; Angius *et al.*, 2016) as showed in Table 5. Lactose was also used to induce expression in substitution of IPTG (Neubauer *et al.*, 1992; Monteiro *et al.*, 2000). Total protein extracts were analysed by SDS-PAGE with Coomassie blue staining. It was expected to find an overexpression band at ≈65 kDa, corresponding to

the Gup1 protein, however, such band was not obtained (representative results in Fig. 1). The absence of Gup1was also verified by Western-Blot using an anti-GFP antibody (not shown).

Table 5 - Incubation conditions tested to optimize the expression of Gup1 in *E. coli.*

Medium	LB and TB
Induction	IPTG (0,1 to 2mM) or lactose (2%)
Temperature	18ºC, 30ºC and 37ºC
Time	2 to 24h

Several causes could explain the inability to produce Gup1 in *E. coli* (for a review see Schlegel *et al.* (2010)). As said above, Gup proteins apparently exist in all eukaryotes, but are missing from prokaryotes. Therefore, Gup1 could suffer fast proteolytic degradation in *E. coli.* Also, *GUP1* might not be translated properly due to deficient codon usage (Bonekamp *et al.*, 1985; Kane, 1995). Gup1 has a large number of residues of arginine, isoleucine, leucine and proline, which could impair expression in *E. coli*, as they are encoded by rare codons in this bacterium. To overcome this problem, the appropriately improved strain BL21-CodonPlus-RIL was used. This strain provides additional copies of rare tRNA genes (the RIL strain carries genes for Arg (AGA and AGG), Ile (AUA), Pro (CCC) and Leu (CUA)). Moreover, this strain is also deficient in Lon and OmT proteases to reduce proteolytic degradation (Jerpseth *et al.*, 1998). Nevertheless, Gup1 was not detected. The reason underlying the unsuccessful Gup1 expression in *E. coli*, despite the high number of conditions and optimizations, remains for now obscure. *E. coli* is considered the best vehicle for heterologous protein expression. Yet, Gup1 is a multispanning membrane protein, which is known to increase the difficulty of heterologous expression (Wang *et al.*, 2003; Wagner *et al.*, 2006). Other unsuccessful attempts to express Gup1 (or just some peptides from this protein) using several expression systems and conditions, are reported (Bleve, 2005; Ferreira, 2005). Bleve (2005) tried to purify Gup1 in *E. coli* BL21-CodonPlus-RIL cells harbouring pET-30a-*GUP1* (Bleve, 2005). Alternatively, selected cytosolic and extracellular membrane domains of Gup1 were cloned in different expression plasmids

(pET-14, pET-24a and pET-42). These were used to transform *E. coli* BL21, Rosetta2 (DE3) and BL21-CodonPlus-RIL (Ferreira, 2005). Given this difficulty, Co-IP was attempted, a protocol useful to detect *in vivo* protein-protein interactions.

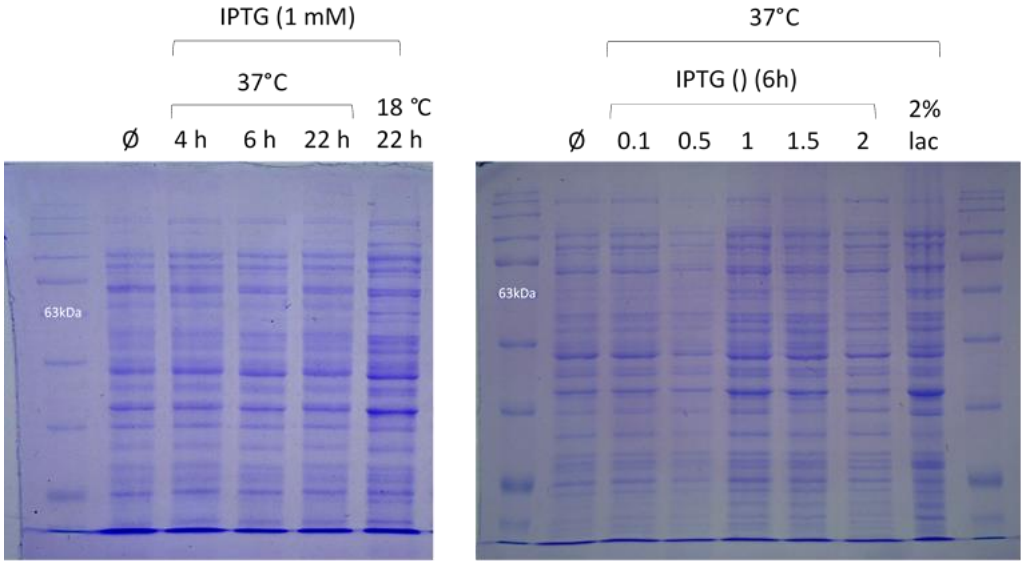

Figure 1 - SDS-Page of *E. coli XL1Blue* harboring pET-25b-*GUP1-6xHIS* construction. The strains were grown in LB medium at 37ºC. Induction conditions were varied: temperature (18, 37°C), inductor (IPTG and lactose (lac)), inductor concentrations (0.1, 0.5, 1, 1.5, 2 mM IPTG), and incubation times (4 – 22h).

Co-IP with Gup1

Co-IP is one of the most straightforward protocols to assess protein-protein interactions, allowing the identification of physiologically valid interactions. Co-IP confirms interactions using a whole cell extract where proteins are present in their native form in a complex mixture of cellular components that may be required for successful interactions. In addition, use of eukaryotic cells enables posttranslational modification which may be essential for interaction and which would not occur in prokaryotic expression systems (Free *et al.*, 2009; Rao *et al.*, 2014). In view of the previous mentioned unsuccessful attempts to purify the protein or parts of it (Bleve, 2005; Ferreira, 2005), anti-yeast Gup1 antibody is not available. Moreover, the recently commercialized anti-HHAT/L antibody designed for higher eukaryotic cells, according to manufacturer information, cannot be utilized in yeasts. Therefore, a chimera Gup1-GFP and anti-GFP antibody were used instead.

Optimization of *GUP1* expression

GUP1-GFP construction cloned in the pYES2 plasmid was used to complement *S. cerevisiae* W303-1A Δ*gup1* mutant. Yeast transformants were grown overnight in YNB-glucose, and the expression induced by changing the culture medium to YNB with galactose 2%. Protein expression was analysed by WB using an anti-GFP antibody at several time points up to 24 h (Fig. 2A). The expression of *GUP1-GFP* was low during the first 4 h of induction and increased afterwards. After 8 h of induction it is possible to see an increase of free-GFP signal corresponding to protein degradation (Fig. 2A). To avoid this, 6 h induction time was chosen to proceed. The proper sub-cellular localization of the chimera Gup1-GFP was assessed by fluorescence microscopy (Fig. 2B). The fluorescence signal localized mainly at the level of the plasma membrane followed by ER (Fig. 2B), agreeing with the previously described primordial sub-cellular localizations of Gup1 protein (Hölst *et al.*, 2000; Bleve *et al.*, 2005), and ensuring that the addition of a GFP-tag does not interfere with the protein location. Fluorescence in mitochondria (Hölst *et al.*, 2000) was not apparent.

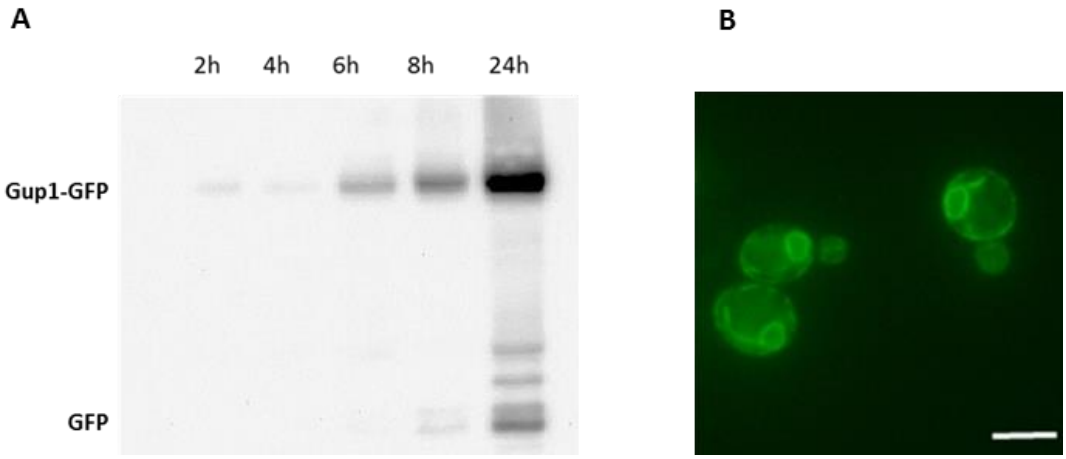

Figure 2 – Expression and localization of Gup1-GFP fusion protein in *S. cerevisiae* W303-1A *Δgup1* transformed with pYES2-*GUP1-GFP*. (A) Western Blot analysis of Gup1-GFP expression using an anti-GFP antibody, after 2, 4, 6, 8 and 24 h of induction with galactose. **(B)** Sub-cellular localization of Gup1-GFP after 6 h of induction by FM. Bar = 5 µm

Optimization of Gup1 solubilization

To proceed to Gup1 immunoprecipitation it was necessary to ensure the solubilization of the protein under native conditions, *i.e.* without inhibiting its function, causing irreversible denaturation, or interfering with the next steps of purification

/identification. Gup1 is a membrane protein (Hölst *et al.*, 2000; Bleve *et al.*, 2005), which is considered a major obstacle for this objective. Usually, a detergent is used to extract and solubilize proteins (Lin and Guidotti, 2009), since it mimics the natural lipid bilayer environment by spontaneously forming micellar structures in which membrane proteins may solubilize. Three commonly used detergents were tested, Triton X-100, Np-40 and CHAPS, at different concentrations and during different periods of solubilization (Fig. 3). The best conditions to solubilize Gup1-GFP consisted in incubating the total cell free extract with 1% NP-40 during 4 h (Fig. 3), as evidenced by the amount of Gup1-GFP that appears in the supernatant of NP-40 treated lysate, when compared to that which remains in the correspondent pellet. As expected from the non-soluble nature of membrane proteins, Gup1 was not found in the supernatant fraction of the untreated lysate (Fig. 3, lane 3).

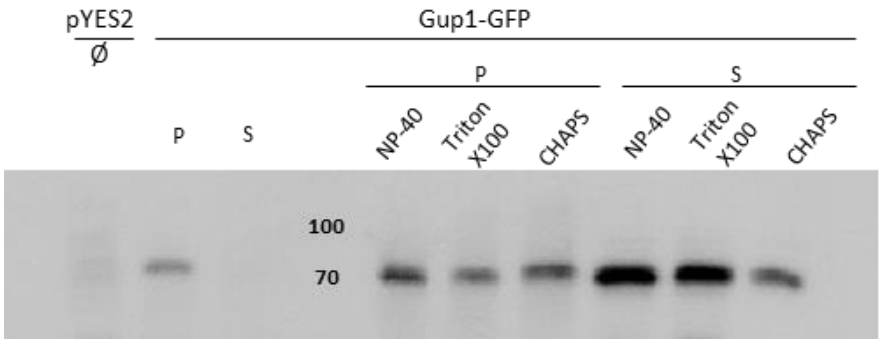

Figure 3 – WB analysis of Gup1-GFP solubility in three different detergents. Δ*gup1* cells transformed with pYES2-*GUP1-GFP* or with the empty plasmid (pYES2Ø) were grown in YNB glucose overnight, and then transferred to YNB with galactose for 6 h to induce protein expression. Cells were collected, lysed and the resultant lysate treated with one of the tested detergents. P = Pellet, S = Supernatant.

Optimization of Co-IP procedures

Co-IP of Gup1-GFP was performed using the Dynabeads Protein G in the solubilized protein lysates of cells expressing this chimeric protein, and analysed in silver or coomassie stained SDS-PAGE. As control for nonspecific protein binding, an immunoprecipitation without antibody was performed. This actually occurred (not shown), therefore, all Co-IP were performed incubating the antibody with the beads, and only afterwards incubating the resultant complex, antibody + magnetic beads, with the sample. This modification enabled to block the nonspecific binding to the beads (not

shown). Other controls consisted in using an immunoprecipitation mixture containing only the antibody (Fig. 4A - lane 2), or the Δ*gup1* cells expressing only GFP (Fig. 4A - lane 3). Several different protein bands were found in the Co-IP using the chimera Gup1-GFP (Fig. 4A). These were extracted and analysed by mass fingerprint and Maldi-TOF (Table 6). Two of these proteins stand out for not having previously been identified as Gup1 partners: Por1 and Pil1 (with ≈30 and ≈38 kDa, respectively). These are two very distinct proteins with well-defined functions in the cell. Por1 is a mitochondrial porin, essential for maintenance of mitochondrial osmotic stability and permeability (Blachly-Dyson *et al.*, 1997). Pil1 is a membrane-associated protein indispensable for eisosomes biogenesis and integrity (Moreira *et al.*, 2009). Plasma membrane was the most recognized Gup1 localization (Hölst *et al.*, 2000; Bleve *et al.*, 2005), while mitochondria localization was suggested by Hölst *et al. (*2000) but was not further confirmed. The fact that Por1 was found through Co-IP suggests it actually occurs. This methodology does not allow the anticipation of how often and in which physiological conditions these interactions may happen.

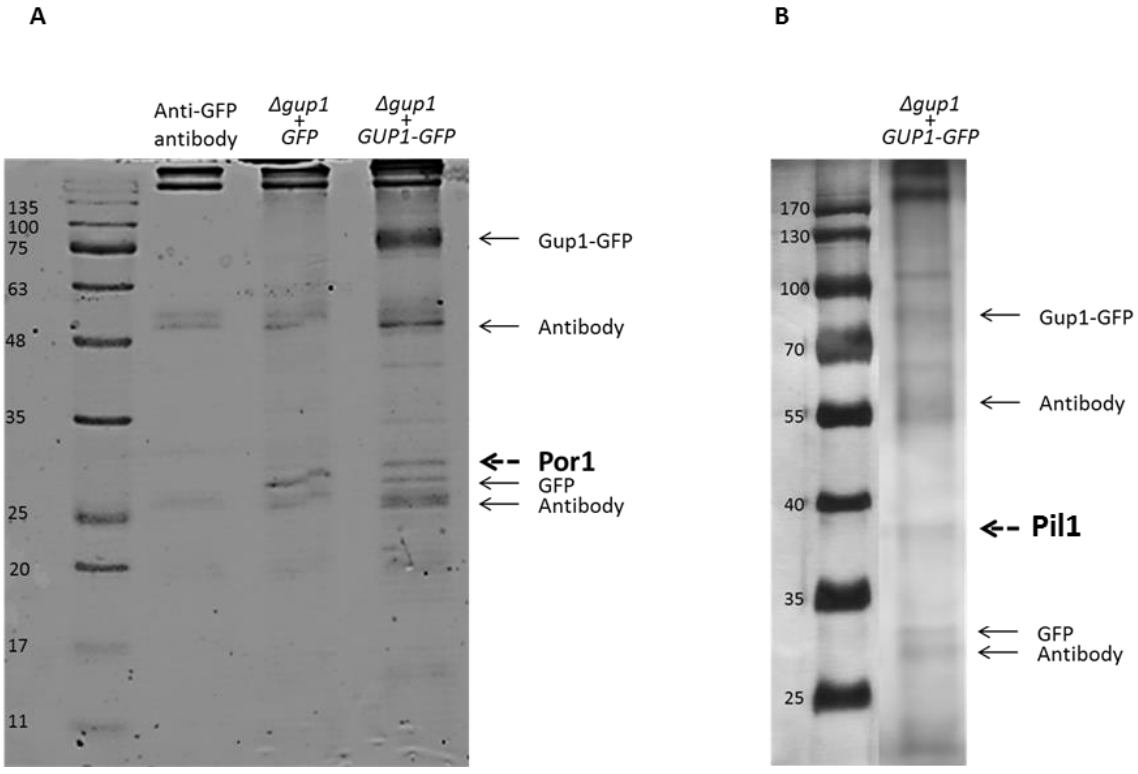

Figure 4 –Co-IP of Gup1-GFP and its partners. Whole cell lysates of *S. cerevisiae* W303-1A *Δgup1* expressing Gup1-GFP were used to immunoprecipitate the Gup1-associated proteins using the capture complex formed by anti-GFP and magnetic beads. The *Δgup1* strain expressing GFP alone was used as control. The co-immunoprecipitated samples were analyzed by SDS-PAGE and the Gup1p-associated proteins found were excised from the gel and identified by LC-MS/MS.

Table 6 - Proteins co-immunoprecipitating with Gup1 identified by mass fingerprint and Maldi-TOF.

Gene Name	Mass (Da)	Score	Sequence coverage (%)	Description
POR1	30524	255	86%	Mitochondrial outer membrane protein porin 1 [*S. cerevisiae*]
PIL1	38319	63	39%	Phosphorylation Inhibited by Long chain bases protein [*S. cerevisiae*]

Conclusions

Several whole genome screenings predicted several putative Gup1 physical partners (Table 1). Only one specific work indirectly identified Mep2 as a physical partner of Gup1 (Van Zeebroeck *et al.*, 2011). This work is the first systematic attempt to identify the physical partners of Gup1. From two approaches: (1) expression in *E. coli* as a preliminary step to affinity chromatography, and (2) Co-IP of Gup1 and its partners in native conditions using specific Gup1 as bait, only the second was successful. Two novel physical partners of Gup1 were found: Por1 and Pil1, which different localization deepens the previous notion that Gup1 is able to localize in several different subcellular fractions and there could perform diverse roles. The nature of these interactions/roles is assassed in the next chapters.

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CHAPTER 3

Mitochondria VDAC (Por1) physical interacts with Gup1

Joana Tulha and Candida Lucas (2017). Mitochondria VDAC (Por1) physical interacts with Gup1. *In preparation*

Abstract

The deletion of *Saccharomyces cerevisiae GUP1* is associated with, namely, plasma membrane and cell wall structure, lipid metabolism, trafficking, cytoskeleton organization and budding pattern, extra cellular matrix composition, and in *Candida albicans* with morphological switching, biofilm formation, virulence and antifungal resistance. Consistently with these multiple intracellular localizations, several proteins were previously described to putatively interact with Gup1, mostly through HTP surveys. Using the co-immunoprecipitation assay, the yeast mitochondria VDAC (Voltage-Dependent Anion Channel) Por1 was identified as a physical partner of Gup1. Por1 is a porin located in the outer mitochondria membrane, required for the maintenance of this membrane permeability, as well as mitochondrial osmotic stability. Accordingly, the co-localization of Por1 and Gup1 in the mitochondrial fraction was confirmed by cellular fractionation followed by Western Blot. It was also observed that the absence of Gup1 seems to affect the cellular levels of Por1, as well as its correct localization.

The double mutant Δ*gup1*Δ*por1* was constructed, and used together with the correspondent single mutants to assess *GUP1* deletion-associated phenotypes. The changed nature of acetic acid-induced cell death observed in the Δ*gup1* mutant, favouring a necrosis-like program, proved to be dependent of the presence of the Por1. Importantly, the association of the two proteins proved to be also important for the control of cell wall integrity, evidenced by changes in the pattern of sensitivity/resistance of the mutants to wall-perturbing agents and high temperature, suggesting a putative interference in the CWI pathway. From the Δ*gup1*-associated morphology phenotypes, *POR1* deletion impacted in the differentiation of structured colonies, and the size of multicellular aggregates/mats. These results are compatible with a role of the Gup1/Por1 pair in the control of cell fate.

Introduction

The *Saccharomyces cerevisiae GUP1* gene encodes a transmembrane-spanning protein that belongs to the membrane-bound *O*-acyltransferase superfamily (MBOAT) (Hofmann, 2000; Neves *et al.*, 2004). Gup1 is implicated in a complex and extensive series of phenotypes involving major cellular processes, such as cytoskeleton polarization (Ni and Snyder, 2001), bud site selection pattern (Casamayor and Snyder, 2002), secretory and endocytic pathways (Bonangelino *et al.*, 2002), telomere length (Askree *et al.*, 2004), cell death (Tulha *et al.*, 2012) and on the extracellular matrix (ECM) protein and sugar fractions composition (Faria-Oliveira *et al.*, 2015a, 2015b). Furthermore, physiological studies using *GUP1* deleted strains have evidenced the involvement of Gup1 in the integrity and composition of the cell wall (Ferreira *et al.*, 2006), lipid membrane composition (Oelkers *et al.*, 2000) and anaerobic sterol uptake (Reiner *et al.*, 2006), the GPI-anchors remodelling (Bosson *et al.*, 2006), and the sphingolipid-sterol ordered domains (lipid rafts) integrity and assembly (Ferreira and Lucas, 2008). The consequent disordered distribution of Pma1 H^+-ATPase (Ferreira and Lucas, 2008) was shown to be responsible for the phenotype on glycerol active uptake that originally allowed the discovery of this protein (Hölst *et al.*, 2000). The Gup1 from the human commensal/pathogen *Candida albicans* was also implicated in the biological processes that underlie virulence, i.e., the capacity of cells to differentiate into hyphae, to adhere and invade, and to correctly form biofilm (Ferreira *et al.*, 2010). Nevertheless, the actual role of Gup1 protein remains elusive. One of the reasons underlying the difficulty in pinpointing the action of this protein comes from the multiple localizations that Gup1 apparently displays: plasma membrane, ER and possibly also mitochondria (Hölst *et al.*, 2000; Bleve *et al.*, 2005). The study of the Gup1 interactome is therefore a topic of great interest.

The yeast mitochondrial VDAC (Voltage-Dependent Anion Channel), Por1, was found to be a physical partner of Gup1 (Chpt. 2). VDAC is a highly-conserved voltage-dependent pore from the outer mitochondrial membrane that functions as a low specificity molecular sieve for small hydrophilic molecules (Colombini, 1979). The channel adopts an open conformation at low membrane potential and a closed conformation at potentials above 30-40 mV (Colombini, 1979). VDAC is, therefore,

very important for the control of mitochondrial membrane permeability (Lee *et al.*, 1998), the preservation of mitochondrial osmotic stability (Sánchez *et al.*, 2001) and the regulation of mitochondrial respiration (Ahmadzadeh *et al.*, 1996; Blachly-Dyson *et al.*, 1997). It facilitates the exchange of ions and molecules, like ATP, ADP, pyruvate, malate, and other metabolites between mitochondria and cytosol (Blachly-Dyson and Forte, 2001). Additionally, this pore is also an important regulator of Ca^{2+} transport (Shoshan-Barmatz and Gincel, 2003). In accordance, VDAC physically interacts with several mitochondrial and cytoplasmic proteins, including the ATP-dependent cytosolic enzymes hexokinase I and II, and glucokinase (Brdiczka, 1990). Thus, it is believed that VDAC can act as an anchor point for proteins, which therefore have an easier access to ATP produced by the mitochondria (Blachly-Dyson *et al.*, 1993).

Several lines of evidence suggest that VDAC participates in mitochondrial outer membrane permeabilization, a determinant step in apoptotic cell death (Desagher and Martinou, 2000). During mammalian apoptosis, increased permeability of VDAC allows the release of apoptogenic factors such as cytochrome *c* (cyt *c*) (Shimizu *et al.*, 1999; Tsujimoto and Shimizu, 2002). Although cyt *c* plays an essential role in oxidative phosphorylation within mitochondria, in the cytosol of mammalian cells it is responsible for a major apoptotic cell death pathway (Jiang and Wang, 2004; Shoshan-Barmatz *et al.*, 2006). The mechanism by which VDAC facilitates the release of cyt *c* from mitochondria, has not yet been fully elucidated. Some authors propose that VDAC might contribute to the opening of a pore – the Permeability Transition Pore – across the mitochondrial membranes, which eventually leads to mitochondria swelling and rupture of the outer mitochondrial membrane (OMM), thus releasing cyt *c* (Szabó and Zoratti, 1993; Szabó *et al.*, 1993). Other authors proposed that oligomerization between individual VDAC subunits, creates a large flexible pore through which cyt *c* can pass (Zalk *et al.*, 2005; Shoshan-Barmatz *et al.*, 2010). VDAC could also associate with Bcl2-family members (a group of mammalian apoptotic regulators) to mediate the permeabilization of OMM (Priault *et al.*, 1999; Arbel *et al.*, 2012). *S. cerevisiae* Por1 was also associated with the regulation of yeast programmed cell death (Pereira *et al.*, 2007; Trindade *et al.*, 2016). Indeed, the deletion of *POR1* increases the sensitivity of Δ*por1* yeast cells to apoptosis inducing conditions (Pereira *et al.*, 2007; Trindade *et al.*, 2016).

Gup1 has also been associated to the cell death processes. The Δ*gup1* mutant from *S. cerevisiae* exhibits hypersensitivity to acetic acid and reduced chronological life span (Tulha *et al.*, 2012), two conditions that have been associated to the induction of apoptosis (Ludovico *et al.*, 2001; Fabrizio and Longo, 2008). Nevertheless, Δ*gup1* cells undergo a necrotic-like cell death process, characterized by the absence of typical apoptotic features, including maintenance of the membrane integrity, phosphatidylserine externalization, depolarization of mitochondrial membrane, and chromatin condensation, all of which are observed in *wt* cells (Tulha *et al.*, 2012).

The present work aims at characterizing the interaction between Gup1 and Por1 in *S. cerevisiae*. For that purpose, a simple phenotype assessment of the correspondent single and double mutants was performed in the attempt to identify the major processes associated with the interaction between the two proteins. These included, first and foremost the response to acetic acid-induced programmed cell death, as well as the survival to cell wall and membrane perturbing agents including high temperature for their association with mitochondria function and associated signalling. Additionally, the mutants were also used to verify the maintenance of other Δ*gup1*-associated phenotypes, namely cellular and colony morphology.

Material and Methods

Strains and growth conditions

The bacteria and yeast strains used in this study are listed in Table 1. *Escherichia coli* XL1 Blue was purchased from Stratagene. Bacteria were cultivated in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl (2% agar for solid medium) pH 7.2) appropriately supplemented for antibiotic resistance when necessary (100 mg/mL ampicillin or 50 μg/mL kanamycin). Cultivation of bacteria, as well as isolation and manipulation of plasmid DNA, were done using standard procedures (Ausubel *et al.*, 1999). Two *S. cerevisiae* genetic backgrounds were used, BY4741 (Euroscarf) and W303-1A (Table 1). Yeasts were cultivated on YPD (1% yeast extract, 2% peptone, 2% glucose, 2 (2% agar for solid medium)), or YNB medium (0.175% YNB without amino acids and nitrogen source (Difco), 0.5% $(NH_4)_2SO_4$, 2% glucose or galactose)

appropriately supplemented according to auxotrophic requirements. Liquid cultures were performed in batch at 30ºC and 200 rpm orbital shaking in a 1/3 air to liquid ration.

Table 1 - Microbial strains used in the present study.

Strain	Genotype	Source
S. cerevisiae W303-1A *wt*	*MATa; leu2-3,112; trp1-1; can1-100; ura3-1; ade2-1; his3-11,15*	Thomas and Rothstein, 1989
S. cerevisiae W303-1A Δ*gup1*	*MATa; leu2-3,112; trp1-1; can1-100; ura3-1; ade2-1; YGL084c::HIS5*	Hölst *et al.*, 2000
S. cerevisiae W303-1A *pYES2Ø*	*MATa; leu2-3,112; trp1-1; can1-100; ura3-1; ade2-1; YGL084c::HIS5 pYES2Ø*	This study
S. cerevisiae W303-1A *pYES2-GFP*	*MATa; leu2-3,112; trp1-1; can1-100; ura3-1; ade2-1; YGL084c::HIS5 pYES2-GFP*	This study
S. cerevisiae W303-1A Δ*gup1 pYES2-GUP1-GFP*	*MATa; leu2-3,112; trp1-1; can1-100; ura3-1; ade2-1; YGL084c::HIS5 pYES2-GUP1-GFP*	This study
S. cerevisiae BY4741 *wt*	*MATa; ura3Δ0; leu2Δ0; his3Δ1; met15Δ0*	Euroscarf
S. cerevisiae BY4741 Δ*gup1*	*MATa; ura3Δ0; leu2Δ0; his3Δ1; met15Δ0; YGL084c::kanMX4*	Euroscarf
S. cerevisiae BY4741 Δ*por1*	*MATa; ura3Δ0; leu2Δ0; his3Δ1; met15Δ0; YNL055c::kanMX4*	Euroscarf
S. cerevisiae BY4741 Δ*gup1*Δ*por1*	*MATa; ura3Δ0; leu2Δ0; met15Δ0; YNL055c::kanMX4; YGL084c::HIS3*	This study
S. cerevisiae BY4741 Δ*por1 - POR1-GFP*	*MATa; ura3Δ0; leu2Δ0; met15Δ0; YNL055C::POR1-GFP-HIS3*	Huh *et al.*, 2003
S. cerevisiae BY4741 Δ*gup1*Δ*por1 - POR1-GFP*	*MATa; ura3Δ0; leu2Δ0; his3Δ1; met15Δ0; YNL055C::POR1-GFP-HIS3; YGL084c::kanMX4*	This study
E. coli XL1 Blue	*endA1gyrA96 (nalR) thi-1 recA1 lac glnV44 F'[::Tn10 proAB+ lacIq Δ(lacZ)M15]hsdR17(rK- mK+)*	Stratagene

Construction of *S. cerevisiae* Δ*gup1*Δ*por1* double mutant and Δ*gup1*Δ*por1-POR1-GFP*

The double mutant Δ*gup1*Δ*por1* of *S. cerevisiae* BY4741 was constructed replacing the *GUP1* gene in BY4741 Δ*por1* (Euroscarf) with the *gup1::HIS3* disruption cassette amplified by PCR from the p416 plasmid (Addgene) with the primers A and B in Table 2. The amplicon thus obtained was used to transform Δ*por1* strain by homologous recombination using standard protocols (Ito *et al.*, 1983). The generated transformants

were selected in YNB medium without histidine. Positive clones were confirmed by colony PCR using the *GUP1* deletion confirmation primers E and F in Table 2.

Table 2 - Primers used in the present study and their sequence.

Name	**Primer**
A - Fw *gup1::HIS3* cassette	5'ATGTCGCTGATCAGCATCCTGTCTCCCCTAATTACTTCCGTTTC CCGCAATTTTCTTTTTC 3'
B - Rv *gup1::HIS3* cassette	5'TCAGCATTTTAGGTAAATTCCGTGCCTCTTTTCTTCTTCTATAT ATATCGTATGCTGCAGC 3'
C - Fw *gup1::KanMx* cassette	5'ATGTCGCTGATCAGCATCCTGTCTCCCCTAATTACTTCCGGAC ATGGAGGCCCAGAATAC 3'
D - Rv *gup1::KanMx* cassette	5'TCAGCATTTTAGGTAAATTCCGTGCCTCTTTTCTTCTTCTCAGT ATAGCGACCAGCATTC 3'
E - Fw *GUP1* deletion confirmation	5' ATCAGCTCAATCGGACATA 3'
F - Rv *GUP1* deletion confirmation	5' ATCATATGGTCCAGAAACC 3'
G - Rv *GUP*1 deletion confirmation	5' CTGCAGCGAGGAGCCGTAAT 3'

The construction of the Δ*gup1*Δ*por1-POR1-GFP* was performed deleting the *GUP1* gene from the Δ*por1-POR1-GFP* strain (kindly provided by Erin K. O'Shea, Howard Hughes Medical Institute, USA (Huh *et al.*, 2003)). The *GUP1* gene was deleted using the KanMx disruption cassette, amplified from pUG6 plasmid (Addgene) with the primers C and D listed in Table 2. The *gup1::KanMx* disruption cassette was used to transform Δ*por1-POR1-GFP* strain by homologous recombination using standard protocols (Ito *et al.*, 1983). Transformants were selected in YNB medium with Geneticin (200mg/L). Positive clones were confirmed by colony PCR using *GUP1* deletion confirmation primers E and F listed in the Table 2.

Co-Immunoprecipitation (Co-IP) assay

S. cerevisiae W303-1A Δ*gup1* was transformed with (i) the pYES2-*GUP1-GFP* plasmid (kindly provided by G. Bleve, ISPA, Unità di Lecce, Italy), (ii) the pYES2Ø, or (iii) with the pYES2-*GFP*. Standard procedures were used (Ito *et al.*, 1983). The expression of Gup1-GFP was induced for 6 hours in YNB medium with 2% galactose. For the Co-IP, 250 mL of Δ*gup1* strain expressing Gup1-GFP were collected, and the

pellet suspended in 1 vol. of lysis buffer (50 mM Tris-HCl, pH 7.5; 100 mM NaCl; 2 mM EDTA) containing proteases inhibitors (0.4 µl/ml aprotinin; 1 µg/ml leupeptin; 1 µg/ml pepstatin; 1 mM PMSF). Cell lysis was accomplished with 4 cycles of 1 min vortexing the cell suspension with 1 vol. 0.5 mm ø glass beads intercalated with 1 min cooling on ice. IP buffer 10x (500 mM Tris-HCl, pH 7.5; 1 M NaCl; 20 mM EDTA; 10% NP-40) was added to cell lysate to a final concentration of 1x, and incubated 4 h at 4ºC with constant mixing. Antibody-conjugated beads (anti-GFP (Roche) - Dynabeads® (Invitrogen)), prepared following manufacturer instructions, were mixed with the cell lysates and incubated overnight at 4ºC. The complex beads-antibody-protein/s were then washed 3 times with IP buffer 1x and, once with PBS 1x (137 mM NaCl; 2.7 mM KCl; 100 mM Na_2HPO_4; 2 mM KH_2PO_4; pH 7.4) using a magnetic stand, resuspended in 50 µL elution buffer (Laemmli buffer) and boiled for 30 min at 70ºC. The resulting co-immunoprecipitated samples were separated by 12% SDS–PAGE, and the proteins were visualized staining with colloidal coomassie blue as previously described (Dyballa and Metzger, 2009). Stained bands were excised and identified by mass spectrometry (Peptide mass Fingerprinting and MALDI-TOF) (Unidad de Proteómica, Parque Científico de Madrid, UCM, Spain).

Isolation of mitochondria and ER-containing microsomal sub-cellular fractions

Overnight cultures of *S. cerevisiae* W303-1A *Δgup1* pYES2-*GUP1-GFP* on YNB glucose (2 L) were collected by centrifuging 5 min at 5,000 rpm, transferred to induction medium YNB galactose and incubated at 30°C for 6 h to obtain Gup1-GFP expression. The culture was then identically collected and cells were converted into spheroplasts by enzymatic digestion with zymolyase (Zymolyase 20T, Seikagaku Biobusiness Corporation). The spheroplasts were disrupted using hand-potter homogenization, and the mitochondrial and ER-containing microsomal fractions recovered after a series of differential centrifugations as previously described (Gregg *et al.*, 2009). ER-containing microsomal fraction was recovered by ultracentrifugation of the post-mitochondrial fraction at 100,000 g. The mitochondrial fraction was purified in a sucrose-gradient centrifugation at 134,000 g (Gregg *et al.*, 2009). Mitochondrial and ER suspensions were frozen in liquid nitrogen and stored at -80°C.

Protein precipitation, SDS-PAGE and Western Blotting

Protein extracts were prepared as previously described (Sivaraman *et al.*, 1997). Proteins were precipitated with 3 M TCA (trichloroacetic acid), washed with acetone, and solubilized in 2x Laemmli buffer (Laemmli, 1978). To confirm the Co-IP, the correspondent samples were separated by 12% SDS–PAGE. To assess Gup1-GFP localization by Western-Blotting, 50 μg of proteins were precipitated, solubilized in 2x Laemmli buffer (Laemmli, 1978) and separated by 12% SDS–PAGE.

Proteins separated by SDS-PAGE were then blotted into PVDF membranes, blocked with 5% milk in PBS containing 0.05% Tween 20, and incubated with primary antibodies: mouse monoclonal anti-GFP (1:3000; Roche); mouse monoclonal anti-Por1 (yeast mitochondrial porin (1:5000, Molecular Probes), mouse monoclonal anti-Pgk1 (yeast cytoplasmic phosphoglycerate kinase) (1:5000, Molecular Probes); and rabbit monoclonal anti yeast-Dpm1 (ER-resident dolichol phosphate mannose synthase) (1:1000; Invitrogen). Membranes were incubated with secondary antibodies against mouse or rabbit IgGs (1:10000) and revealed by chemiluminescence (ECL+Amersham) according to manufacturer instructions.

Total RNA isolation

Yeast samples for real-time PCR analysis (~$5x10^7$ cells) were collected and the cell pellets were mechanically disrupted using 0.5 mm ø glass beads in a swing-mill at 30 Hz for 15 min. Total RNA was extracted and isolated using the NucleoSpin® RNA kit (Macherey-Nagel), and subsequently quantified in a ND-1000 UV-visible light spectrophotometer (NanoDrop Technologies). RNA quality was evaluated by agarose-gel electrophoresis. The absence of contaminant gDNA was verified by directly using the isolated RNA as template in real-time PCR assays (RNA not reverse-transcribed to cDNA).

Quantitative Real Time-PCR (qRT-PCR)

Primers for qRT-PCR (Table 3) were built using Primer3Plus software, aligned against *S. cerevisiae* genome sequence (http://www.yeastgenome.org/blast-sgd) for specificity confidence, and analysed with the Mfold server

(http://unafold.rna.albany.edu/?q=mfold) to check for the possible formation of self-folding secondary structures. Total RNA (500 µg) was reverse-transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad). The cDNA levels were then analysed using the Bio-Rad® CFX96 Touch™ real-time PCR instrument. Each sample was tested in duplicate in a 96-well plate (Bio-Rad, CA). The reaction mix (10 µL final vol.) consisted of 5 µL of SsoAdvanced™ SYBR® Green Supermix (Bio-Rad), 0.25 µL of each primer (250 nM final concentration) and 1 µL of cDNA preparation. A blank (No Template) control was included in each assay. The thermocycling program consisted of one hold at 98°C for 30 sec, followed by 40 cycles of 10 sec at 98°C and 20 sec at 60°C. After completion of these cycles, a melting-curve was performed (65°C-95°C; 0.5°C increments, 3s) and data collected to verify PCR specificity, contamination and the absence of primer dimers. Three different extractions of total RNA were analysed, by at least duplicate PCRs. The data were normalized to *18S* gene. The comparative Ct method analysis ($2^{-\Delta\Delta CT}$ method) (Schmittgen and Livak, 2008) was used to analyse the results. The results presented are the mean of the three different RNA extractions.

Table 3 - qRT-PCR primers used in the present study and their sequence.

Name	**Primer**
Fw *GUP1* qRT-PCR	5' GCGTGGGAAAATGACACAC 3'
Rv *GUP1* qRT-PCR	5' AAACAGCCTCCACGGAATC 3'
Fw *POR1* qRT-PCR	5' TGGCGCAGAGTTTGGTTAC 3'
Rv *POR1* qRT-PCR	5' GTTCAATGTAGCGCCCAAG 3'
Fw *18S* qRT-PCR	5' TGCGATAACGAACGAGACC 3'
Rv 18S qRT-PCR	5' TCAAACTTCCATCGGCTTG 3'
Fw *PGK1* qRT-PCR	5' TGGTGGTGGTATGGCTTTC 3'
Rv *PGK1* qRT-PCR	5' TTTCAGCACCAGCCTTGTC 3'

Microscopy procedures

Fluorescence microscopy. To verify the sub-cellular localization of GFP, as well as sub-cellular chitin distribution through Calcofluor White (CFW) staining, a Leica Microsystems DM-5000B epifluorescence microscope with the appropriate filter

settings using a 100x oil-immersion objective was used. Images were acquired through a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software.

Light microscopy. Cell morphology was observed by light microscopy (LM) in mid-exponential yeast cultures. Microscopy assessments were done in a Leica Microsystems DM-5000B epifluorescence microscope. Images were acquired through a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software. For observe colony morphology/differentiation, mid-exponential yeast cultures were diluted 100x, spotted (50 µL) on YPD (1% glucose), and incubated for 12 days at room temperature. Resulting colonies were visualized in a Leica Zoom 2000 stereo microscope and the images were acquired through a Leica EC3 digital camera and processed with LAS AF Leica Microsystems software.

Sedimentation and stress phenotypes

Culture sedimentation. To assess the sedimentation phenotype, cells were grown to mid-exponential phase in YPD at 30ºC. Cultures were then collected to a final $O.D._{600}$=1, in a micro-tube and left to rest at room temperature for 20 min, after which the resultant sedimentation was photographed.

Drop tests. Drop tests were performed using identical $O.D._{600}$=1 cell suspensions at a final $O.D._{600}$=1, also collected from mid-exponential YPD-grown cultures. Four 10-fold serial dilutions were made, and 5 µL of each suspension was applied on respective medium. Results were scored after 3 days of incubation at 30ºC, unless otherwise stated.

Mat formation

The ability of yeast strains to form a mat was assessed as previously described (Reynolds and Fink, 2001), with some modifications. Overnight cultures were collected by centrifugation, diluted to a final $O.D._{600}$=1 in water, and 5 µl of this suspension was used to inoculate 0.3% agar YPD plates (all plates were prepared at the same time (one day before) to ensure the same level of medium hydration). The plates were then sealed with parafilm and incubated at room temperature. Results were scored after 12 days of incubation by measuring the diameter of the mat.

Effect of acetic acid on yeast viability

Yeast strains were grown until mid-exponential phase ($O.D._{600}$ at 0.6–0.8) on YNB medium, after which they were collected and resuspended to a final $O.D._{600}$=0.2 in fresh YNB adjusted to pH 3.0 with HCl, and containing 150 mM acetic acid (T_0). Incubation took place for 180 min at 30°C. At determined time points, 40 µL 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 the number of c.f.u. obtained at T_0.

Quantification of PI staining by flow cytometry

Flow cytometry was used to assess membrane integrity by counting the cells stained with propidium iodide (PI) (Sigma Aldrich). Cells were harvested, washed and resuspended in PBS containing 4 µg/mL PI. The samples were incubated for 10 min at room temperature in the dark, and analysed in an Epics® XL™ (Beckman Coulter) flow cytometer. At least 20,000 cells from each sample were analysed.

Results and Discussion

Confirmation of Por1 as a physical partner of Gup1

In order to identify the molecular partners of Gup1, a Co-IP assay was performed. The pYES2-*GUP1-GFP* plasmid was used to transform a W303 Δ*gup1*, and the expression of the chimeric protein Gup1-GFP induced for 6 h in YNB galactose. The proper cellular localization of the chimera Gup1-GFP was confirmed by fluorescence microscopy. Co-IP was performed using the Dynabeads Protein G in the protein-solubilized lysates of cells expressing the chimeric protein. As controls, the immunoprecipitation mixture containing only the antibody was used, as well as a protein preparation of Δ*gup1* cells with a pYES2-*GFP* plasmid, expressing GFP alone. For further details on procedures please consult Chpt. 2.

From the several bands obtained exclusively in the Co-IP sample of the strain expressing chimeric Gup1-GFP, the one corresponding to a molecular weight of 30 KDa was identified as Por1, the mitochondrial VDAC (Voltage-Dependent Anion Channel). Subsequently, WB using a specific anti-Por1 antibody was performed in the previously obtained Co-IP samples (using anti-GFP antibody as bait). As can be seen in Fig. 1, the Por1 was only detected in the Co-IP sample of the strain expressing chimeric Gup1-GFP and not in the negative control (cells expressing GFP alone). This result clearly confirmed the physical interaction between Gup1 and Por1.

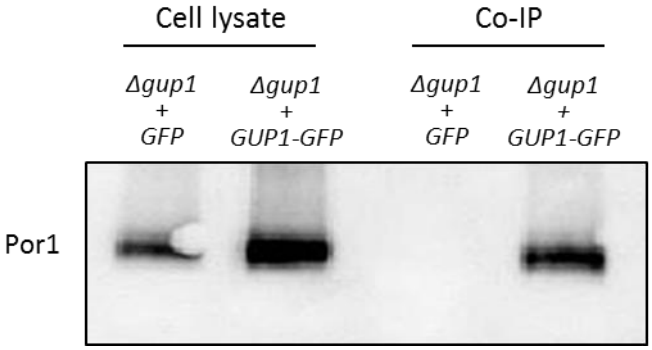

Figure 1 – Co-Immunoprecipitation (Co-IP) of Gup1-GFP and its partners. Whole cell lysates of *S. cerevisiae* W303-1A Δ*gup1* expressing Gup1-GFP were used to immunoprecipitate Gup1-associated proteins using the capture complex formed by anti-GFP and magnetic beads. The Δ*gup1* strain expressing GFP alone was used as control. Co-immunoprecipitated samples, as well as total precipitated cell lysates, were analyzed by WB using anti-Por1 antibody. One representative experiment is shown.

Co-localization of Gup1 and Por1 in the mitochondrial fraction

Gup1 was previously clearly associated with the plasma membrane and the endoplasmic reticulum (ER) (Hölst *et al.*, 2000; Bleve *et al.*, 2005), but possibly also with the mitochondrial sub-cellular fraction (Hölst *et al.*, 2000). Yet, fluorescence of the Gup1-GFP construct was not perceptive in the mitochondria (Chpt. 2 – Fig. 2). Therefore, the putative co-localization of Gup1 and Por1 in yeast mitochondria was investigated. Cellular fractionation of the Δ*gup1* strain expressing Gup1-GFP was performed, and the mitochondrial, ER and cytosolic fractions were purified. The localization of Gup1-GFP chimera was determined by WB, using an anti-GFP antibody. To ensure the purity of these fractions, specific antibodies for well-known proteins that

characterize each fraction were used (Fig. 2): Por1 for mitochondria, Dpm1 (dolichol phosphate mannose synthase) for ER, and Pgk1 (phosphoglycerate kinase) for cytosol.

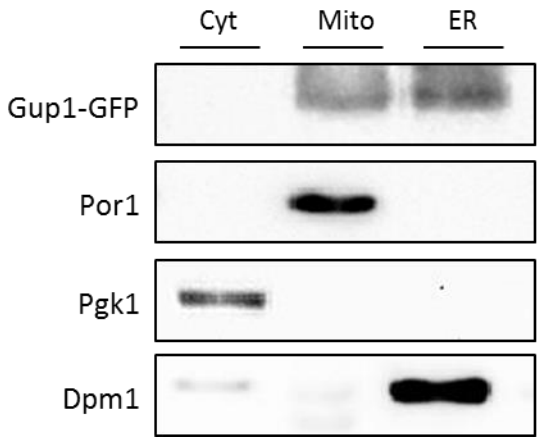

Figure 2 – Sub-cellular localization of Gup1 in *S. cerevisiae* W303-1A Δ*gup1* expressing a Gup1-GFP chimera. The purified cytosolic (Cyt), mitochondrial (Mito) and microsomal-containing ER fractions were analyzed by WB using an anti-GFP antibody. Antibodies against Por1, Pgk1 and Dpm1 were used to control the purity of mitochondrial, cytosolic and ER fractions, respectively. These proteins are considered molecular markers of each of these sub-cellular fractions. One representative experiment is shown.

As expected from the results of Hölst *et al.* (2000), a band corresponding to Gup1-GFP was observed in the ER and mitochondrial fraction, but not in the cytosol (Fig. 2). This result confirms that Gup1 and Por1 co-localize in the mitochondrial sub-cellular fraction. The actual co-localization of the two proteins in the outer mitochondrial membrane, where Por1 is reported to exist, remains to be seen in the future. Moreover, since Gup1 is present in the ER, another possibility is that these proteins interact in a region called ER-Mitochondria Encounter Structure (ERMES) where the ER and mitochondria form a junction (Kornmann *et al.*, 2009). This structure integrates components with several functions, such as the maintenance of mitochondrial morphology, protein biogenesis and Ca^{2+} binding, and is involved with the regulation of lipid metabolism and transport, bioenergetics, autophagy and apoptosis. Importantly, a proteomic analysis of ERMES has confirmed the therein presence of Por1 (Stroud *et al.*, 2011), and Por1 was shown to actually interact with several ERMES-complex subunits, including Mdm12, Mdm34 and Mmm1 (Kornmann *et al.*, 2011; Murley *et al.*, 2015). Still, the presence of Gup1 in this specific region was not reported.

POR1 expression, protein levels and localization in the Δ*gup1* mutant

To evaluate if the absence of Gup1 interfered with *POR1* expression, this was quantified by RT-PCR. *POR1* expression was quantified relative to the ribosomal subunit *18S*, and compared with the relative expression level in the corresponding *wt* strain. Results show that the expression of *POR1* in the Δ*gup1* mutant is slightly higher than in the *wt* strain (Fig. 3A). However, WB analysis of Por1 levels in these strains revealed approximately 50% less Por1 protein in the Δ*gup1* strain (Fig. 3B). This was done by tagging *POR1* with GFP under the regulation of its own promoter in the *wt* and Δ*gup1* backgrounds, and quantifying the levels of Por1-GFP densitometrically in comparison with the constitutive protein Pgk1 (phosphoglycerol kinase 1). As control, the expression of *PGK1* in the *wt* and Δ*gup1* strains was also quantified by RT-PCR, and was identical in both strains (not shown). Therefore, the difference found between the *POR1* mRNA and Por1 is not an artefact but a real effect of *GUP1* deletion, direct or indirect.

An increase in gene expression does not result *per se* in a higher protein level. Several factors can influence the ultimate levels of a protein in the cell, the most common of which is protein (or organelle) degradation. The reduced levels of Por1 in Δ*gup1* cells might indicate a reduction of the total mitochondrial mass in this mutant, which could result from an increase in mitophagy (process of selective degradation of mitochondria via autophagy). Several *GUP1* deletion-associated phenotypes suggested the involvement of this protein in CWI and/or HOG signalling pathways, which are known to be related with the induction of mitophagy in yeast (Aoki *et al.*, 2011; Mao *et al.*, 2011). On the other hand, overexpression of Gup1 was previously demonstrated to induce the proliferation of intracellular membranes containing ER and Golgi resident proteins (Bleve *et al.*, 2011). Nevertheless, no information is known about the consequences of *GUP1* over-expression or disruption in the total mitochondrial mass. Since a chimera Por1-GFP is being used, an increase in protein/organelle degradation should yield GFP degradation visible by WB, which was not the case (not shown). Additionally, the accumulation of GFP in the vacuole, as a result of Por1-GFP degradation, was not observed by fluorescence microscopy (Fig. 3C). In view of these results, protein degradation (or mitophagy) seems not to be the reason for the lower levels of Por1 in the Δ*gup1* mutant. Other processes can also affect the cellular levels of a protein, from transcription to post-translational modifications, or even secretion. In a

previous work from our group, characterizing the proteome from the extracellular matrix (ECM) of a *S. cerevisiae* biofilm-like mat (Faria-Oliveira *et al.*, 2014, 2015b), Por1 was found in both *wt* and Δ*gup1* ECM. On the other hand, in liquid batch cultures, Por1 was only found in the growth medium of Δ*gup1* cultures, providing a possible explanation for the fact that much less protein is found in Δ*gup1*. These results might indicate that Gup1 is influencing the route that the Por1 protein follows, eventually leading to its selective secretion according to solid or liquid growth conditions.

Por1 localization in the *wt* strain and Δ*gup1* mutant was analysed using the strains expressing the chimeric Por1-GFP (Fig. 3C). As expected, we observed that Por1 exhibits clear mitochondrial localization in *wt* cells, being the green florescence distributed in patches in the mitochondrial networks. On the other hand, in the Δ*gup1* mutant, the localization of Por1 follows a more homogeneous distribution in mitochondria. To understand if this derives from an altered mitochondrial morphology, the mitochondrial network of Δ*gup1* was observed using two different mitochondrial probes: mitotracker red and $DiOC_6$. The Δ*gup1* mitochondrial morphology is similar to that of *wt* cells (not shown; Tulha *et al.*, 2012), therefore, the miss-localization of the Por1 in the mutant is not a secondary effect of abnormal mitochondrial morphology, but rather an effect of the absence of Gup1.

The change in the levels and/or localization patterns of proteins in the absence of Gup1 is not a new result. This was already observed for various proteins, namely the plasma membrane ATPase Pma1, the GPI-anchored Gas1p (Ferreira and Lucas, 2008), and the Mep2 ammonium transporter, the only protein described to physically interact with Gup1 in a no HTP work (Van Zeebroeck *et al.*, 2011). Additionally, Gup1p has an important role in the assembly/integrity of lipid rafts (Ferreira and Lucas, 2008), which can cause, at least in some cases, the miss-localization of plasma membrane proteins as the above-mentioned. The presence of rafts in the mitochondria is still a controversial topic, however, several lines of evidence have suggested their existence (Mollinedo, 2012), though their composition is still unknown. It could be that mitochondria membrane rafts are similar to the plasma membrane rafts, and identically exhibit altered stability/assembly in the absence of Gup1, this way promoting the miss localization of Por1. Still, in view of the many variables poised by the complex biogenesis pathway that Por1 assembly requires, which includes the TOM and SAM machineries, and depends on cardiolipin (Gebert *et al.*, 2009; Endo and Yamano, 2010; Schmidt *et al.*,

2010; Dukanovic and Rapaport, 2011; Becker *et al.*, 2013), this interpretation remains speculative, for the time being.

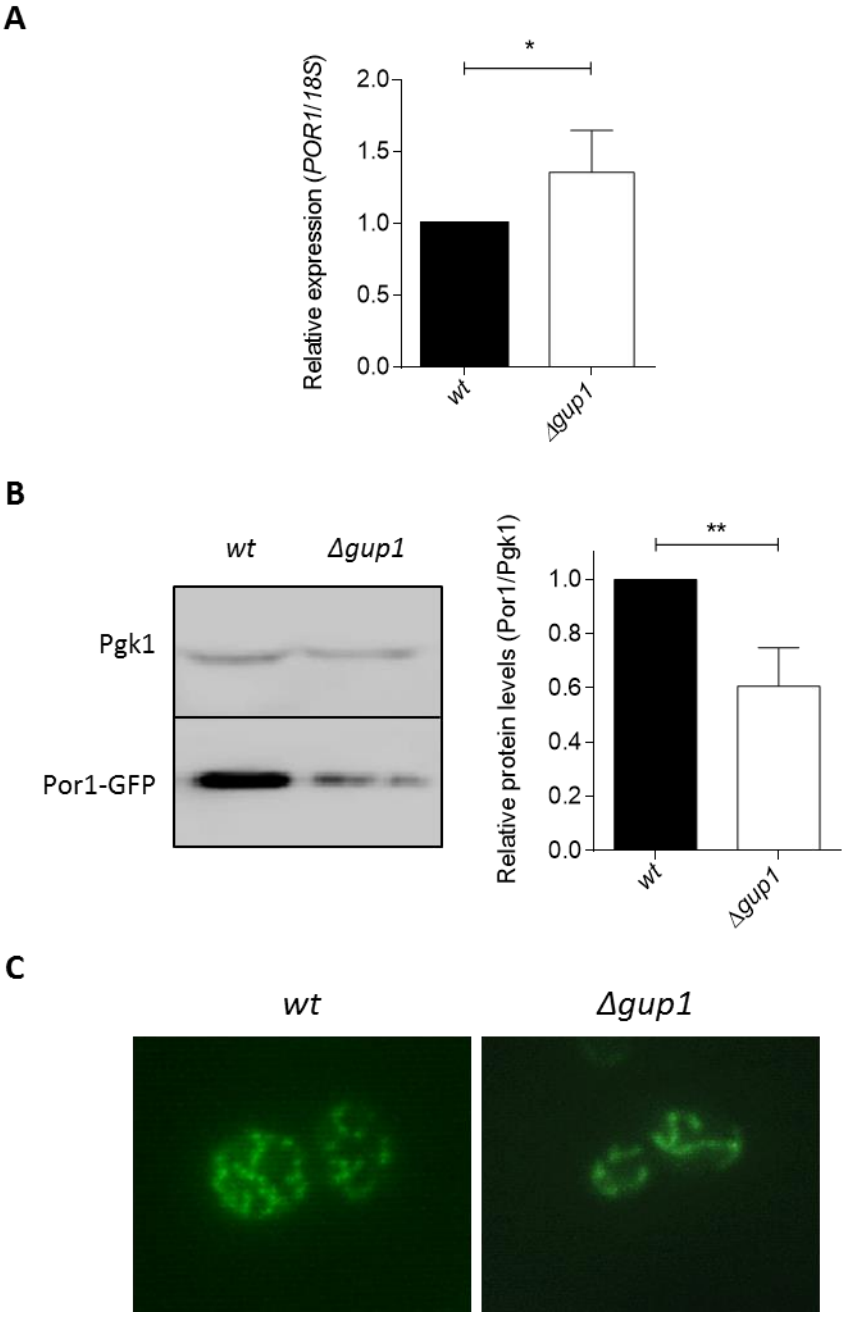

Figure 3 – *POR1* expression (A), protein levels (B) and localization (C) in *S. cerevisiae* BY4741 *wt* and *Δgup1* strains. Cells were grown in YNB glucose medium until mid-exponential phase. **(A)** Relative expression of *POR1* assessed by qRT-PCR. *POR1* expression was normalized against the ribosomal subunit 18S and represented relative to the levels in the *wt* strain as calculated by the comparative Ct method analysis ($2^{-\Delta\Delta CT}$ method). **(B)** The levels of Por1-GFP in whole cell protein extracts was assessed by WB using anti-GFP and anti-Pgk1 antibodies (left panel). Densiometric quantification of Por1 levels was also preformed (right panel). The relative densitometry units were normalized to Pgk1 protein levels using Image J software. **(C)** The Por1 localization was assessed using the *wt* and Δ*gup1* strains with a chromosomal Por1-GFP insertion. Results are representative of at least three independent experiments. **P < 0.01; *P < 0.05 t-test.

Effects of Por1 and its interaction with Gup1

To better assess the biological functions associated to the Gup1 and Por1 interaction, the double mutant Δ*gup1*Δ*por1* was generated. This construction was performed replacing the *GUP1* gene with a *HIS3* disruption cassette in the BY4741 Δ*por1* background from Euroscarf collection. The resulting double mutant was then tested regarding several physiological, morphological and signalling processes, chosen according to the phenotypes previously described to characterize Δ*gup1*. The single deleted strains in the same genetic background were also evaluated. Assays included mitochondria-related phenotypes such as cell death in response to acetic acid stress, and the consumption of respired versus fermented carbon sources. Moreover, the response to other stress conditions well-known to inflict less or more severe damage in the Δ*gup1* strain was also assessed, including cell wall damaging drugs, temperature and osmotic stress. Additionally, cell and colony morphology were verified.

Acetic acid-induced cell death process

Acetic acid triggers an apoptotic-like cell death in *S. cerevisiae*, with characteristics similar to mammalian apoptosis (Ludovico *et al.*, 2001). G*UP1* deleted mutant was found to be more sensitive to acetic acid-induced cell death, though dying with features of a non-apoptotic cell death (Tulha *et al.*, 2012). This was concluded based on the absence of several apoptotic markers (i) plasma membrane integrity, (ii) phosphatidylserine externalization, (iii) depolarization of mitochondrial membrane, and (iv) chromatin condensation. The response of the double mutant Δ*gup1*Δ*por1* to acetic acid was tested and compared with identically assayed single mutated strains (Fig. 4A). In agreement with previous observations, the single deletions of *GUP1* (Tulha *et al.*, 2012) or *POR1* (Pereira *et al.*, 2007; Trindade *et al.*, 2016) increased the sensitivity of yeast cells to acetic acid (Fig. 4A). Both Δ*gup1* and Δ*por1* mutants exhibited a reduction in survival after 3 h of exposure to acetic acid when compared to *wt* cells. This phenotype is more evident in Δ*por1* than in Δ*gup1* cells (displaying respectively ±15% and ±25% survival). Interestingly, the simultaneous absence of Gup1 and Por1 completely reversed the sensitivity of these strains to acetic acid (Fig. 4A), being the percentage of survival of the Δ*gup1*Δ*por1* strain similar to that of *wt* cells (±45% and ±50%, respectively).

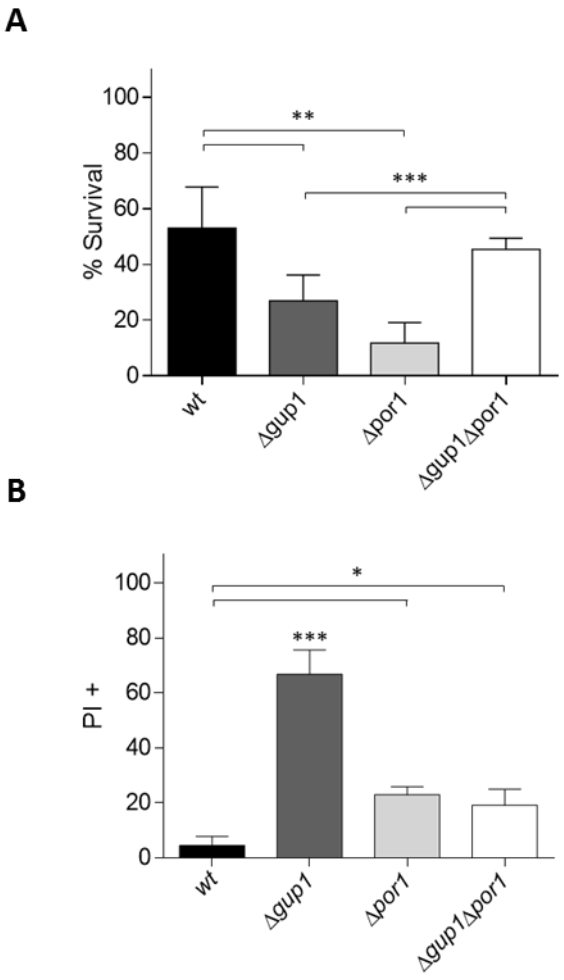

Figure 4 – *S. cerevisiae* BY4741 *wt, Δgup1, Δpor1* and *Δgup1Δpor1* strains response to acetic acid-induced cell death. Exponentially growing cells were treated with 150 mM acetic acid for 3h. **(A)** Viability was determined by c.f.u. assay (results were normalized with 100% survival corresponding 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 ± SD of at least 3 independent experiments. ***P < 0.001; **P < 0.01; *P < 0.05 one-way ANOVA followed by Tukey's test.

Staining the cells with PI and analysing by flow cytometry is a procedure usually used to assess membrane integrity, and serves as preliminary assay to discriminate between an accidental or programmed cell death types. The number of PI+ cells was quantified under the same experimental conditions used to assess the effect of acetic acid on viability (Fig. 4B). As expected, the decrease of viability of *wt* (Ludovico *et al.*, 2001) and Δ*por1* (Pereira *et al.*, 2007; Trindade *et al.*, 2016) cells were not accompanied by a correspondent increase in loss of plasma membrane integrity, which suggests cells are dying by apoptosis. Δ*gup1* also behaved as expected (Tulha *et al.*,

2012). The decrease in cell survival was accompanied by an extensive loss of plasma membrane integrity (Fig. 4B), indicating a non-apoptotic type of cell death. On the other hand, the Δ*gup1*Δ*por1* mutants behaved very similar to *wt*, the decrease in cell survival not being accompanied by an equivalent increase in PI+ cells, which did not exceed 20% (Fig. 4B).

As mentioned before, *GUP1* deletion changes the nature of acetic acid-induced cell death in yeast, favouring a necrosis-like program over a more controlled process (Tulha *et al.*, 2012). The present work clearly shows that this result depends on the presence of the Por1, since (i) the sensitive phenotype of the Δ*gup1* mutant to acetic acid-induced cell death was almost totally reversed when both proteins are absent, and (ii) the loss of plasma membrane integrity (indicative of a necrotic cell death) observed in Δ*gup1* cells is not observed in the double mutant Δ*gup1*Δ*por1*. This suggests that Gup1 and Por1 do interact in the regulation of programmed cell death in a way that requires to be assessed in the future.

In mammalian cells, VDAC was suggested to contribute to mitochondrial membrane permeabilization, through a still unknown mechanism, leading to the release of apoptogenic factors, such as cyt *c*. It was assumed that VDAC was one of the components of the mitochondrial Permeability Transition Pore (PTP), together with ANT (equivalent to yeast ADP/ATP carrier (AAC)) and Cyp-D (equivalent to yeast Cpr3p) (McEnery, 1992; Crompton *et al.*, 1998). However, recent studies suggested otherwise, that VDAC proteins are actually not essential components of the mammalian PTP (Kokoszka *et al.*, 2004; Krauskopf *et al.*, 2006; Baines *et al.*, 2007). Accordingly, Por1 seems to be dispensable for the formation of a PTP in *S. cerevisiae*, as well (Lohret and Kinnally, 1995), although a possible regulatory role of this protein in PTP opening is not entirely discarded (Gutiérrez-Aguilar *et al.*, 2007; Trindade *et al.*, 2016). Por1 has been associated with the regulation of programmed cell death, presumably working as a negative regulator of the apoptotic response. Indeed, the Δ*por1* cells are more sensitive to apoptotic inducing conditions presenting an increase in cyt *c* release (Pereira *et al.*, 2007; Trindade *et al.*, 2016).

It was also suggested that yeast Por1 might have other physiological functions (Owsianowski *et al.*, 2008). For example, evidence supporting a role for Por1p in the organization of the mitochondrial network was found (Trindade *et al.*, 2016). Accordingly, in mammalian cells, VDAC was implicated in the association of

mitochondria with the cytoskeleton (Lindén and Karlsson, 1996). Disruption of the interaction of mitochondria with the cytoskeleton alters the normal mitochondrial morphology, giving origin to a fragmented mitochondrial network (Boldogh *et al.*, 1998), which could contribute to an increased susceptibility to apoptosis-inducing conditions, *e.g.* by facilitating the release of apoptotic factors. Gup1 was described as being involved in cytoskeleton organization (Ni and Snyder, 2001). Moreover, this protein is involved in other cellular processes, such as cell wall composition, assembly, stability and morphology (Ferreira *et al.*, 2006), membrane rafts integrity and assembly (Ferreira and Lucas, 2008), lipid metabolism (Oelkers *et al.*, 2000; Bosson *et al.*, 2006; Ferreira and Lucas, 2008), and telomere length (Askree *et al.*, 2004), all of which can be directly or indirectly related with apoptosis. Therefore, from the present work it becomes clear that Por1 and Gup1 are key participants in the response to programmed cell death in yeast. It remains to be clarified in the future whether and how these two proteins interact and work, if they share or not the same pathway in the regulation of yeast cell death, and if the phenotypes above mentioned are consequences of that interaction.

Growth on different carbon sources and at high temperature

Mitochondria function is metabolically crucial for *S. cerevisiae*. Not only is it associated to the consumption of exclusively respired carbon sources in alternative to glucose, like glycerol or ethanol, it is also associated to the consumption of glucose itself. *S. cerevisiae*, as a Crabtree-positive species, ferments and respires glucose at the same time (van Urk *et al.*, 1989). The regulation of important key metabolic pathways that allow cellular homeostasis in terms of *e.g.* redox potential or ATP availability, is only possible when mitochondria are functioning regularly. Otherwise, *S. cerevisiae* strains harbouring defective mitochondria develop a well-known phenotype called *petite*. In this case, the colonies are small sized, which originated their designation, due to their inability to produce ATP by oxidative phosphorylation. *Petite* yeasts are more sensitive to high temperatures than their *wt* relative (Zubko and Zubko, 2014). The temperature sensitivity phenotype is further associated with an altered membrane and/or cell wall composition/integrity (de Nobel *et al.*, 2000).

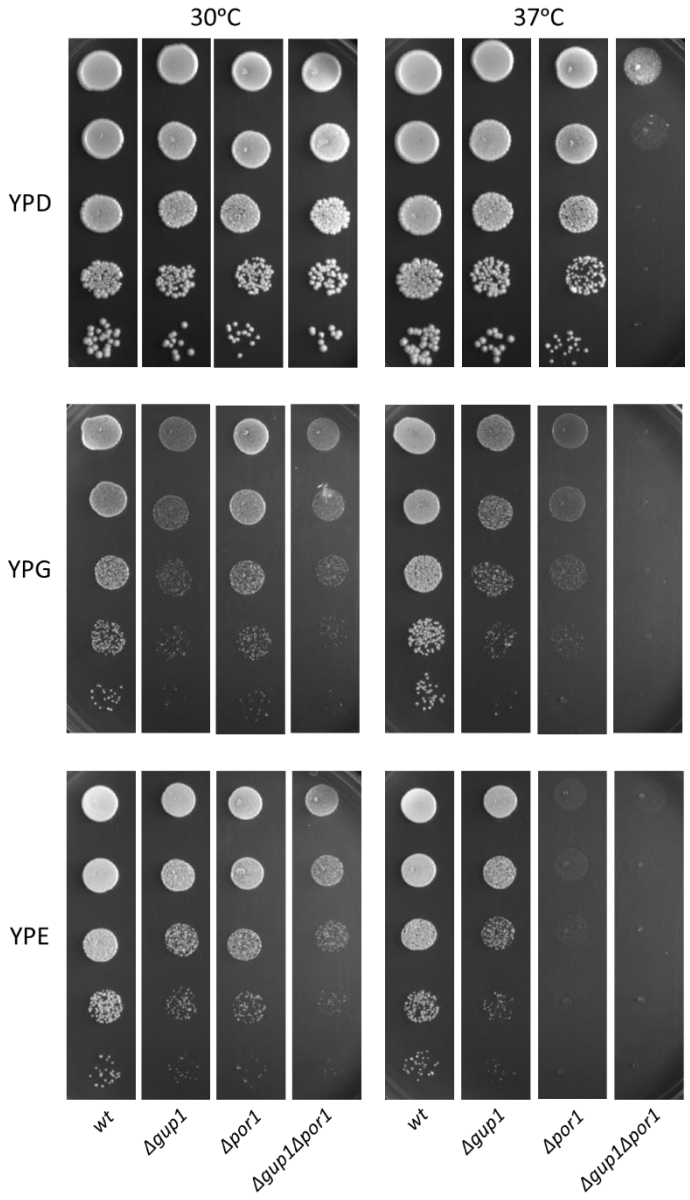

Figure 5 – Carbon source and high temperature-related phenotypes of *S. cerevisiae* BY4741 *wt*, *Δgup*1, *Δpor1* and *Δgup1Δpor1* strains. Strains were grown on YPD until O.D.$_{600}$=1 and 10-fold serial dilutions of each culture were spotted on YP 2% agar medium with 2% (w/v) glucose (YPD), glycerol (YPG) or ethanol (YPE). Results were scored after 3 days incubation at 30˚C or 37˚C. One representative experiment is shown.

The effect of carbon source and high temperature on the Por1/Gup1 pair was verified. *S. cerevisiae wt, Δgup1, Δpor1* and Δ*por1*Δ*gup1* were cultivated at 30ºC or 37ºC, on solid medium supplemented with glucose (fermentation conditions), ethanol or glycerol (respired carbon sources) (Fig. 5). Both *GUP1* and *POR1* individual deletions exhibited a small decrease in growth ability on non-fermentable carbon sources, at 30°C

(Fig. 5, left panels), more pronounced in the Δ*gup1* mutant growing on glycerol. These results are identical to previously reported phenotypes for Δ*gup1* (Ferreira *et al.*, 2006), and for Δ*por1* (Blachly-Dyson *et al.*, 1997; Sánchez *et al.*, 2001). On the other hand, the growth of the double deleted mutant at 30°C on glycerol was identical to Δ*gup1*, and more pronouncedly affected than in either of the single mutants, on ethanol. At 37ºC these phenotypes were aggravated (Fig. 5, right panels). The Δ*gup1* mutant, as previously reported (Ferreira *et al.*, 2006), was more sensitive to high temperature than the *wt* in both glycerol and ethanol. In glucose, the temperature sensitive phenotype of this mutant was not so obvious in this work, perhaps due to the different genetic background used. Accordingly, other studies have reported different responses in growth rate and stress response between W303 and BY4741 genetic backgrounds (Cohen and Engelberg, 2007).

The Δ*por1* was more seriously affected, growing much less on glycerol, and not at all on ethanol, as was the case of the double mutant on either non-fermentable substrate. Actually, this last mutant grew poorly also on YPD, suggesting that the double deletion increases the sensitivity to high temperatures. These results suggest that most probably the mitochondrial function is compromised when both *POR1* and *GUP1* are deleted in spite that neither strain forms *petites*. The fact that the double mutant is almost non-viable at 37°C could indicate, besides the involvement of mitochondria, the existence of severe phenotypes at the level of cell wall structure and/or biogenesis, as well.

Cell wall stress-related phenotypes

The Δ*gup1* mutant grows less at 37 than at 30°C (Ferreira *et al.*, 2006), and concomitantly presents severely altered plasma membrane and cell wall composition and structure (Ferreira *et al.*, 2006). To test whether the deletion of Por1 also introduced any change in this regard, cells of the Δ*por1* and double deleted mutant were exposed to several well-known cell wall-perturbing agents: (i) CR and CFW (Ram and Klis, 2006), which bind to chitin that way interfering with proper cell wall assembly (Roncero and Durán, 1985), and (ii) caffeine, that indirectly activates the CWI pathway through the TORC1 protein kinase complex (Lum *et al.*, 2004; Kuranda *et al.*, 2006). Furthermore, SDS detergent that affects membrane stability (Igual *et al.*, 1996) was also used. Additionally, cells were further incubated with 1M sorbitol that is known to remediate growth defects associated with cell wall instability by osmotically stabilizing the

damaged cells (Levin, 2011). In Fig. 6A, the left panels represent the phenotypes caused by these drugs, and the right panels the possible remediation of the obtained phenotypes by sorbitol.

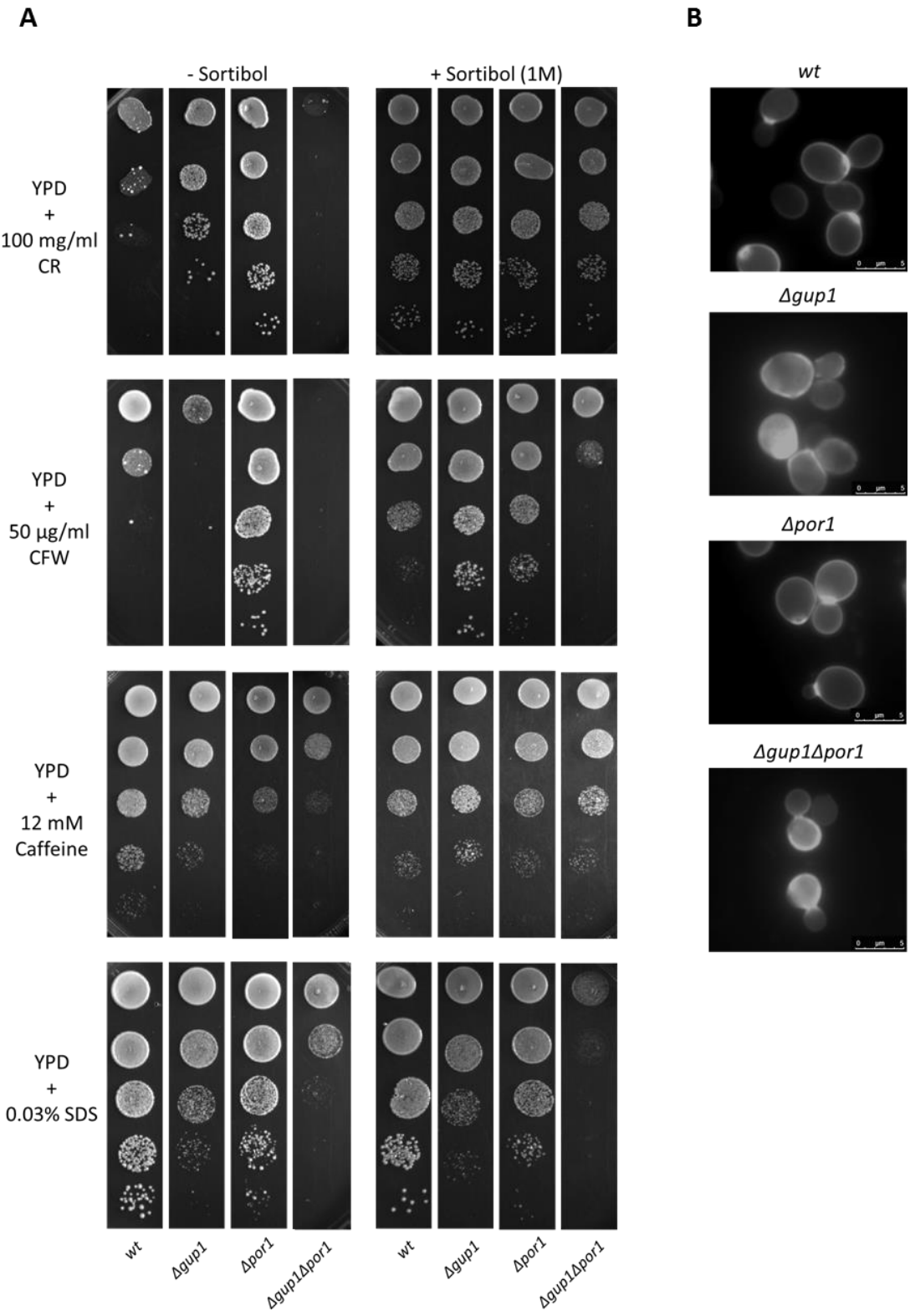

Figure 6 – Membrane and cell wall stress-related phenotypes of *S. cerevisiae* BY4741 *wt, Δgup1, Δpor1* and *Δgup1Δpor1* strains. (A) Cells were grown on YPD until $O.D._{600}$=1 and 10-fold serial dilutions of each culture were spotted on different media. Results were scored after a 3 days incubation at 30˚C. **(B)** CFW staining of mid-exponential YPD grown cells visualized by FM. One representative experiment is shown.

The sensitivity phenotypes observed for Δ*gup1* mutant were all consistent with a primary defect in cell wall biogenesis as well as altered membrane composition in accordance with the literature (Ferreira *et al.*, 2006; Ferreira and Lucas, 2008). The Δ*por1* resisted CR and CFW but displayed a sensitive phenotype when exposed to caffeine, suggesting that Por1 might be somehow contributing for the CWI signalling. Importantly, the double deleted mutant was much more severely affected, especially by CR and CFW that totally impaired growth. The aggravation of the wall-related phenotypes in the double mutant, in comparison with the Δ*gup1*, strongly suggests that Por1 and Gup1 interaction, but not Por1 by itself, implicates in the cell wall integrity and biogenesis, as well as the associated signalling, at least through the CWI pathway.

This is not the first time that a mitochondrial protein is implicated with the cell wall biogenesis. For instance, the Δ*pgs1* mutant, lacking the phosphatidylglycerol phosphate synthase (Pgs1), has a defective cell wall due to decreased β-1,3-glucan, which leads the authors to propose that the deficiency in mitochondrial anionic phospholipid synthesis impairs cell wall biogenesis (Zhong and Greenberg, 2005). Still, the defective wall from the Δ*gup1* has ±30% more β-1,3-glucans, as well as twice the chitin of the *wt* (Ferreira *et al.*, 2006), from which it can be inferred that a defective cell wall can derive from different causes. To our knowledge, the involvement of Por1 in processes related to membrane and/or cell wall was never documented, and the process by which it may impact the wall remains to be found in the future.

Sorbitol remediation occurred (Fig. 6A - right panels) but was not enough to restore full growth of the double mutant in the presence of CFW, in opposition to CR treated cells. This concurs with these compounds not operating through the same path (Fig. 6A). As mentioned above, CFW and CR preferentially interact with carbohydrates in the yeast cell wall such as chitin (Herth, 1980; Pringle, 1991; Kopecká and Gabriel, 1992). As a result, the cell wall becomes weakened, which stimulates the cell wall stress response (Levin, 2011) (i) by the activation of genes encoding proteins that have cell wall-reinforcing functions (Boorsma *et al.*, 2004), and (ii) by the consequent increased

deposition of chitin in the cell wall (Klis *et al.*, 2002; Ram *et al.*, 2004). Thus, it is not surprising that most cell wall mutants have more chitin in their walls, becoming more sensitive to these compounds (Roncero and Durán, 1985; Roncero *et al.*, 1988; Ram *et al.*, 2004; Imai *et al.*, 2005). This is the case of Δ*gup1,* as mentioned above, that has a weak cell wall with twice the amount of chitin compared to *wt* and displays great sensitivity to CFW (Ferreira *et al.,* 2006). Still, Δ*gup1* is not identically sensitive to CR, indicating that chitin levels in the cell wall are not the only factor determining CR sensitivity, and that CFW and CR might induce at least partially separate responses as suggested above. In accordance, the transcriptional profiles induced by CR and CFW (Kuranda *et al.*, 2006) are significantly different. CFW treatment induces a large spectrum of gene expression alteration (altering expression of genes from functional categories like RNA metabolism, transport, organelles biogenesis and response to stress). CR treatment induces a smaller gene expression alteration, being the differentially expressed genes restricted almost exclusively to the cell wall remodelling category (Kuranda *et al.,* 2006). Moreover, the effect of CFW on Δ*gup1* mutant could result from a possible malfunction on HOG pathway, since CFW, unlike CR, could trigger both HOG and CWI/PKC pathways (Alonso-Monge *et al.*, 1999; García-Rodriguez *et al.*, 2000).

Por1, on the other hand, is a mitochondrial protein for which, to our knowledge and as above mentioned, there is no information regarding any kind of cell wall-related phenotype. For this reason, and to observe the chitin distribution on the cell wall, the single and double mutant strains were chitin-stained with CFW (Fig. 6B). The *wt* and the Δ*por1* cells presented a uniform distribution of chitin on the cell wall, with a regular accumulation in the bud septum and scars, unlike the Δ*gup1* mutant, in which the distribution of chitin is not uniform and the fluorescence is more intense, consistently with the increased chitin content (Ferreira *et al.*, 2006). The double mutant showed results identical to Δ*gup1*. By one side this suggests that the increase in chitin observed in Δ*gup1* mutants is also underlying the phenotype observed in the double mutant, but on the other hand it is not enough to justify the growth impairment in the presence of CFW or CR. Other cell wall-related processes besides chitin imbalance must be disrupted by the absence of the two proteins.

Subsequently, the phenotype caused by SDS detergent-induced membrane disruption was also analysed. This detergent can cause indirectly disturbances in the cell wall

(Igual *et al.*, 1996). Δ*gup1* displayed, as before (Ferreira *et al.*, 2006), increased sensitivity to 0.03% SDS (Fig. 6A – left panels). This result was even more evident in the double mutant Δ*gup1*/Δ*por1*. Sorbitol did not change these phenotypes (Fig. 6A – right panels), which is in accordance with a membrane-only effect of this detergent (Igual *et al.*, 1996). The Δ*por1* and *wt* strains were in turn insensitive to SDS. In Δ*gup1*, the membrane composition is altered, exhibiting a reduced content of phospholipids and elevated levels of diacylglycerols and triacylglycerols (Oelkers *et al.*, 2000). In accordance, Gup1 interferes in sterol and sphingolipids synthesis (Ferreira and Lucas, 2008), and is involved with the GPI anchor remodelling (Bosson *et al.*, 2006). The Δ*gup1* mutant is also affected in lipid rafts integrity and assembly (Ferreira and Lucas, 2008). The involvement of Gup1 in lipid metabolism and rafts integrity could be the reason underlying its SDS sensitivity.

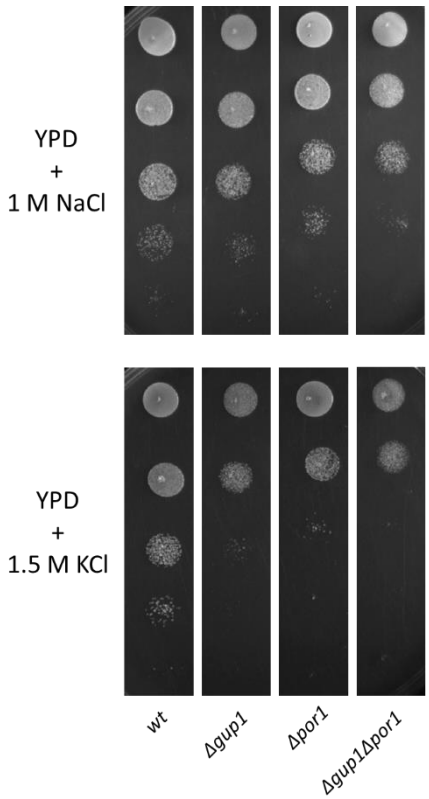

Figure 7– Osmotic stress phenotypes of *S. cerevisiae* BY4741 *wt*, *Δgup1*, *Δpor1* and *Δgup1Δpor1* strains. Cells were grown on YPD until O.D. $_{600}$=1 and 10-fold serial dilutions of each culture were spotted on YPD + 1 M NaCl or YPD + 1.5 M KCl. Results were scored after 3 days incubation at 30˚C. One representative experiment is shown.

The integrity of the cell wall is necessary to survive various kinds of stress that not only high temperature or specific drugs. High osmotic stress for example may become lethal if the cell wall is fragile. Gup1 was long sought to be important for high osmotic stress survival (Hölst *et al.*, 2000; Ferreira *et al.*, 2006). The single and double mutants were thus cultivated in the presence of 1 M NaCl or 1.5 M KCl (Fig. 7). All mutants were equally more sensitive to these stressors than the *wt* strain, suggesting that Por1 is also necessary for osmotic stress response, but the interaction between the two proteins does not add any further/stronger effect.

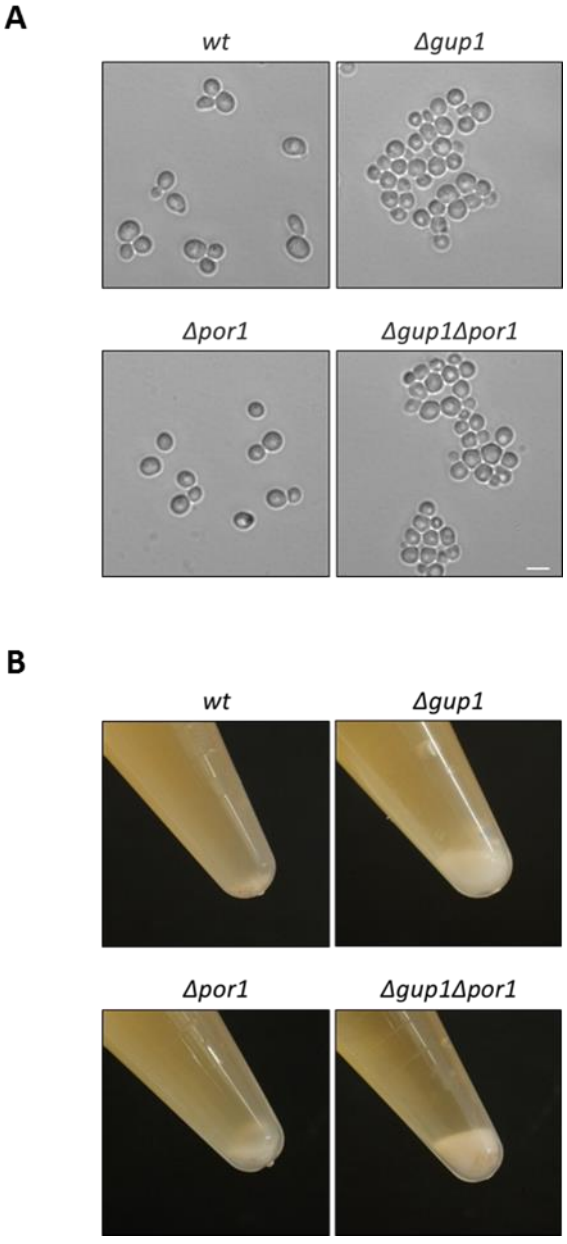

Figure 8 – Cell aggregation and sedimentation of *S. cerevisiae* BY4741 *wt, Δgup1, Δpor1 and Δgup1Δpor1* strains. The assays were performed with mid-exponential cells grown in YPD. **(A)** Cell aggregation was visualized by light microscopy. **(B)** Sedimentation phenotype was recorded after letting the culture rest at room temperature for 20 min. One representative experiment is shown. Bar = 5µm.

Finally, the cell wall also implies other less commonly assessed phenotypes. Cells with specific cell wall composition tend to flocculate or form numerous cellular aggregates. The Δ*gup1* mutant was previously reported to form large and heavy aggregates that swiftly sediment through a process that does not follow any of the known flocculation mechanisms (Ferreira *et al.*, 2006). The present results show that the Δ*gup1* mutant preserved the cell aggregation (Fig. 8A) and depositing (Fig. 8B) phenotypes, not observed in the *wt* or Δ*por1* strains. The double mutant Δ*gup1*Δ*por1,* on the other hand, behaved identically to Δ*gup1* indicating that the sedimentation phenotype is independent of the presence/absence of Por1.

Morphology-related phenotypes

The deletion of *POR1* was not enough to cause a *petite* phenotype. Colonies were identically sized in comparison with *wt* and the other mutants. *S. cerevisiae* laboratory *wt* strains, grown in favourable conditions, form smooth regular-shaped/roundish colonies. When starved for one or more nutrients, or stressed in any way, growth pattern is altered and complex structured colonies may be formed (Granek and Magwene, 2010). A simple methodology was chosen to test the ability of the mutants to differentiate into complex multicellular structures, by carbon-starving the cells for 12 days in solid YPD with only 1% dextrose (Granek and Magwene, 2010). This method had proven efficient with several laboratory and wild yeast strains. In the present case, colonies formed either by *wt*, Δ*gup1* or Δ*por1* exhibited identical moderately irregular-shaped colonies (Fig. 9A), which tended to get more irregular along time. On the other hand, the double mutant Δ*gup1*Δ*por1* presented smooth and uniform round colonies even after 12 days (Fig. 9A).

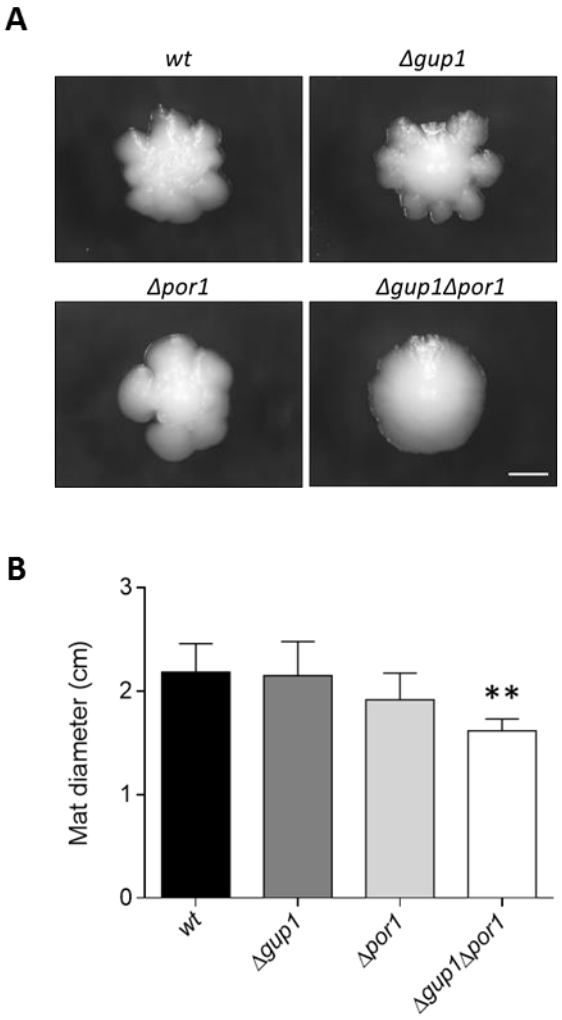

Figure 9 – Colony morphology and mat formation. (A) The colony morphology was visualized after a 10 days growth period in solid YPD (1% dextrose). One representative experiment is shown. Bar = 1mm. **(B)** For mat formation, overnight cultures were inoculated on YPD 0.3% agar plates, and results were scored, after 12 days of incubation at RT, measuring the mat diameter. Results are the mean of at least 3 independent experiments. **P < 0.01 one-way ANOVA followed by Tukey's test.

The expression of the GPI-anchored flocculin *FLO11*, is essential for the formation of structured colonies (Granek and Magwene, 2010; St'ovíček *et al.*, 2010; Vopálenská *et al.*, 2010). Flo11 is important for cell–cell and cell-surface adhesion, and required for pseudo-hyphal differentiation, biofilm formation, colony morphology, and flocculation (Lambrechts *et al.*, 1996; Reynolds and Fink, 2001; Ishigami *et al.*, 2004; Verstrepen *et al.*, 2004). Its expression is lowered in most laboratory strains due to a mutation in the *FLO8* transcriptional activator responding to the RAS/cAMP/PKA signalling pathway (Liu *et al.*, 1996), which explains the dull morphology of colonies formed by laboratory strains when compared to wild yeasts. Still, the deletion of *FLO11* resulted in relatively

few pronounced changes in gene expression, the exceptions being: genes involved in respiration (mitochondria, respiratory chain, ion homeostasis and oxidation/reduction), and genes encoding cell surface proteins (Voordeckers *et al.*, 2012). If Δ*gup1*Δ*por1* caused the total abolishment of *FLO11* expression it could cause the smooth colony phenotype, although this hypothesis needs confirmation.

Similarly to other microorganisms, *S. cerevisiae* is capable of forming multicellular aggregates attached to solid surfaces (e.g., colonies, stalks and mats/biofilms), when growing at a liquid/air interface (e.g., cell films on the surface of sherry wine that are called "flors") or when they mutually interact in a liquid environment and form very large clumps of cells called "flocs" (Engelberg *et al.*, 1998; Reynolds and Fink, 2001; Palková and Váchová, 2006; Soares, 2011; Faria-Oliveira *et al.*, 2014). This last corresponds to the flocculation processes briefly mentioned above. Each of these structures posses some level of internal cell organization and complexity connected with the formation of differentiated cell subpopulations (St'ovíček *et al.*, 2010). Usually, structured colonies tend to produce an abundant ECM and to form biofilms easier and faster (Baillie and Douglas, 2000; Kuthan *et al.*, 2003; Beauvais *et al.*, 2009; Zara *et al.*, 2009; St'ovíček *et al.*, 2010). Previously published data showed a clear influence of the deletion of *C. albicans GUP1* in the ability to develop biofilms (Ferreira *et al.*, 2010). In *S. cerevisiae,* Δ*gup1* produced biofilm-like mats with a sludgy texture (Faria-Oliveira *et al.*, 2015b), although no differences in rate or total amounts produced were described (Faria-Oliveira *et al.*, 2015b). In order to easily compare the ability of the several *S. cerevisiae* strains to colonize spatially in a biofilm-like manner, forming large multicellular aggregates, the production of a different kind of mats (Reynolds and Fink, 2001; Cullen, 2015) was tested. These originate from a controlled number of cells that grow radially in soft agar. Unlike a colony, the surface of this type of agar is colonized radially and extensively and does not require adhesion of the cells to the support surface (Reynolds and Fink, 2001; Cullen, 2015). Therefore, quantifying the diameter of a mat in a fixed period of time provides a comparable indication of the biofilm-producing ability of a given strain (Reynolds and Fink, 2001; Cullen, 2015). The capacity of these strains to form and develop a mat was compared, by cultivating them in a low agar medium (0.3%) during 12 days, and measuring the mat diameter. The Δ*gup1*Δ*por1* mutant displayed a reduced mat diameter when compared to *wt* or the single mutants (Fig. 9B). The reduced mat diameter could indicate a lesser ability to produce and

secrete ECM, though this was not quantified, and is consistent with the duller colonies formed by this strain. These results indicate that the interaction between Gup1 and Por1 proteins is important/essential for the differentiation of structured colonies and the production of more complex and bigger multicellular aggregates.

Conclusions

Several studies demonstrated that Gup1 is involved in a wide range of crucial processes for cell preservation and functioning (Lucas *et al.*, 2016). However, the exact biochemical nature of Gup1 function(s) remains elusive. In this work, a novel *ScGUP1* physical partner was found, Por1, the mitochondrial porin/VDAC. Results of co-localization suggest that Gup1/Por1 interaction could occur in the mitochondria. Still, the possibility that Gup1 could be localized in the ERMES region (since the Gup1 ER localization was already confirmed) cannot be discarded. The deletion of *GUP1* decreases Por1 total protein levels but not *POR1* mRNA expression, which could derive from Por1 increased secretion, previously described (Faria-Oliveira *et al.*, 2015b). Additionally, the deletion of *GUP1* affects Por1 normal distribution on the mitochondrial membrane. The miss-localization of plasma membrane proteins in the absence of Gup1 was already described and was shown to relate with membrane rafts formation. The misdistribution of a mitochondrial membrane protein in Δ*gup1* is here described for the first time. Whether it relates to putative mitochondrial membrane rafts or with ERMES integrity remains to be seen in the future.

In order to perform a phenotypic characterization of Gup1-Por1 interaction the double mutant Δ*gup1*Δ*por1* was generated. The single and double mutants were challenged by diverse physiological conditions previously described as affecting the Δ*gup1*. Differences in the response of the double mutant Δ*gup1*Δ*por1* in relation to the single mutants allowed to identify the common associated processes, and thus, the relevance of the interaction between both proteins. The deletion of both *GUP1* and *POR1* inhibits the formation of complex colonies and delays the formation and development of mats. Moreover, though Δ*por1* cells exhibit, like *wt* and unlike Δ*gup1*, apoptotic cell death in response to acetic acid, the double absence of *GUP1* and *POR1* clearly reverses de single mutants' susceptibility to this apoptotic inducer. In addition,

the double deletion also reverses the massive loss of plasma membrane integrity exhibited by Δ*gup1* cells. Moreover, the Δ*gup1*Δ*por1* also displays extreme sensitivity to high temperatures and to cell wall perturbing drugs, in spite of the resistant phenotype presented by Δ*por1* strain. Finally, Por1 does not appear to interfere with other Gup1-related phenotypes like formation of cell aggregates and sedimentation. The results from this work reveal the importance of the interaction between Gup1 and Por1 in the control of programmed cell death and cell wall integrity, which nature and mode of action remain to be studied in the future.

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CHAPTER 4

Eisosomes component Pil1 physical interacts with Gup1

Abstract

Gup1 is an *O*-acyltransferase that was firstly involved with glycerol active uptake and latter associated with numerous cellular processes including, plasma membrane and cell wall composition, structure and maintenance, lipid metabolism (including GPI anchors remodelling), and rafts integrity. Moreover, Gup1 was also associated with the polarity establishment/bud site selection, secretory/endocytic pathway functionality and vacuole morphology. Still, the exact function of this protein remains unknown. In high Eukaryotes, the Gup1 homologue, HHATL, is responsible for the negative regulation of the Hedgehog (Hh) palmytoilation, and consequently morphogen secretion. In yeast, some proteins were previously identified as physical partners of Gup1 but essentially by whole genome screenings. In this work, a novel protein interacting physically with Gup1 is described – Pil1. This is a membrane associated protein that, together with Lsp1, forms the eisosome core structure. Eisosomes are membrane structures that create invaginations in the plasma membrane and concentrate several proteins, lipids and signalling molecules. Their biological roles are still not completely studied, but they were known to function as cellular reservoirs of lipids, to participate in stress response and to contribute for endocytosis.

It was verified that, in the absence of Gup1, the number of eisosome structures is reduced when compared to *wt*, though Pil1 expression was not affected. Instead, Gup1 may be required for the correct Pil1 assembly in the membrane, through a process that could be dependent of the phosphoinositide levels. The absence of Pil1 did not induce susceptibility to wall disturbing agents/conditions, neither alone or in combination with *GUP1* disruption. In opposition, Pil1 apparently is more important for membrane stability, as the absence of this protein cause susceptibility to SDS, a membrane disturbing agent. Such sensitivity is even more severe in when Gup1 was not present, suggesting important changes on Δ*gup1*Δ*pil1* plasma membrane, possible related to the Gup1-Pil1 interaction.

Introduction

A Co-IP assay (Chpt. 2) identified two possible molecular partners of Gup1. One was the yeast VDAC (Por1), addressed in Chpt. 3, and the other was the eisosome component Pil1 (where Pil stands for "Phosphorylation is Inhibited by Long chain bases"). This is a highly abundant peripheral membrane-associated protein that constitutes, along with Lsp1, the structural core of eisosomes (Zhang *et al.*, 2004; Walther *et al.*, 2006). Eisosomes ("eis" meaning "into" or "portal", and "soma" meaning "body") are large, heterodimeric, immobile protein complexes connected to the cytoplasmic side of specific membrane locations known as MCCs (Membrane Compartment of Can1) (Walther *et al.*, 2006). High-resolution electron microscopy of *Saccharomyces cerevisiae* cells showed that eisosome domains correspond to invaginations of the plasma membrane that form furrows, about 200 to 300 nm long and 50 nm deep (Strádalová *et al.*, 2009). These are formed by filaments of Pil1 and Lsp1 that bind electrostatically with negatively charged lipids in the membranes through their BAR domains (Olivera-Couto *et al.*, 2011; Ziółkowska *et al.*, 2011).

The BAR designation comes from the proteins Bin, Amphiphysin and Rvs (for a review see Ren *et al.*, (2006) and Frost *et al.*, (2009)). BAR domains are composed of three long α-helices that dimerize to form a banana-shaped module with a positively charged concave surface. Cationic residues on the positive surface of this domain interact with anionic membrane lipids via electrostatic interactions, acting as molecular scaffolds that bind to and bend lipid membranes (Frost *et al.*, 2009). BAR domains superfamily is composed of three different families: the N-BARs, the F-BARs, and the I-BARs. In N-BAR and F-BAR dimers, the positively charged concave face is the membrane-binding interface. In contrast, the convex face of I-BAR dimers mediates membrane binding (Frost *et al.*, 2009). The BAR-domain present in the eisossomal Pil1 protein is structurally more similar to the N-BAR domain, that is also found in amphiphysins (Olivera-Couto *et al.*, 2011; Ziółkowska *et al.*, 2011). This domain is capable of self-assembling, and binds lipid membranes, preferably those containing phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P2), allowing membranes to bend and modulating their curvature degree and orientation (Kabeche *et al.*, 2011; Karotki *et al.*, 2011; Olivera-Couto *et al.*, 2011; Suarez *et al.*, 2014). Pil1 BAR domain is, therefore,

essential for protein binding to the membrane and, consequently, for the normal eisosome assembly/organization (Karotki *et al.*, 2011; Olivera-Couto *et al.*, 2011; Ziółkowska *et al.*, 2011). In mammalian cells, BAR domain proteins were implicated in an extraordinary diversity of cellular processes, including fission of synaptic vesicles, cell polarity, endocytosis, regulation of the actin cytoskeleton, transcriptional repression, signal transduction, apoptosis, secretory vesicle fusion, excitation-contraction coupling, cell and tissue differentiation, ion flux across membranes, and tumour suppression (Ren *et al.*, 2006). Some of these processes, like cell polarity, endocytosis, regulation of the actin cytoskeleton, apoptosis and cell differentiation are directly or indirectly affected by the *GUP1* deletion (Ni and Snyder, 2001; Bonangelino *et al.*, 2002; Ferreira *et al.*, 2010; Tulha *et al.*, 2012). Interestingly, in view of the phenotypes long associated with Gup1, it is not surprising that the amphiphysins Rvs167 and Rvs161 were identified as putative genetic interactors (synthetic lethal) of Gup1 (Tong *et al.*, 2004). Yeast Rvs161 and Rvs167 proteins are homologues of the human amphiphysin that also possess a BAR domain. These proteins interact with each other through this domain, and regulate polarization of the actin cytoskeleton, endocytosis, cell polarity, cell fusion, and viability following starvation or osmotic stress (Crouzet *et al.*, 1991; Sivadon *et al.*, 1995; Brizzio *et al.*, 1998; Gammie *et al.*, 1998; Youn *et al.*, 2010).

Eisosomes were initially thought to mark sites of endocytosis (Walther *et al.*, 2006), however, more recent studies demonstrated that most endocytosis occurs through other membrane compartments known as MCP - Membrane Compartments of Pma1 (Grossmann *et al.*, 2008; Brach *et al.*, 2011). Nevertheless, a stable eisosome structure at the cell cortex seems to be required for efficient endocytosis, indicating that the involvement of eisosomes, whose primary function remains uncertain, cannot be completely discarded (Murphy *et al.*, 2011). The MCP microdomain is proposed to associate laterally with MCC (eisosomes) and MCT (Membrane Compartments of TORC2) microdomains, and is defined as the plasma membrane regions containing readily diffusible proteins that are excluded from the other 2 domains (MCC and MCT) (Malínská *et al.*, 2003; Grossmann *et al.*, 2008; Malinsky *et al.*, 2010). While the generally accepted plasma membrane model depicts microdomains as distinct and non-overlapping structures, recent evidences suggest that microdomains might exist in a more interdependent relationship than formerly suspected. One such evidence is the

above-mentioned connection between MCC-associated eisosomes and endocytosis, which occurs at the MCP. Moreover, loss of Tor2 function provokes a reduction in the concentration of synaptojanins and amphiphysins at the endocytic, leading to inefficient scission during endocytosis (Tenay *et al.*, 2013). Together, these evidences indicate that MCC- and MCT-associated proteins influence a cellular process that takes place at the MCP. This concept has influenced a new vision of a more intervening structure and function of the plasma membrane that is still being elucidated.

MCC/eisosome domains have been proposed to participate in several processes, including protecting membrane proteins from endocytosis, as mentioned before (Grossmann *et al.*, 2008), functioning as tension-dependent membrane reservoirs for rapid expansion of plasma membrane (Kabeche *et al.*, 2015a), and maintaining phosphoinositide homeostasis, particularly PI(4,5)P2 (Fröhlich *et al.*, 2014; Kabeche *et al.*, 2014, 2015b). Furthermore, this last function of eisosomes is connected to signalling by CWI pathway (Kabeche *et al.*, 2015b) and the conserved TOR2 complex (Berchtold *et al.*, 2012; Kabeche *et al.*, 2014). The Δ*gup1* mutant exhibits several severe CWI and TORC2 related phenotypes. The cell wall composition, assembly, stability and morphology are affected in the absence of *GUP1*, even though the CWI pathway is working properly in this mutant, as demonstrated by the dual phosphorylated state of Slt2 upon induction by a hypo-osmotic shock (Ferreira *et al.*, 2006). Furthermore, many of the cellular functions controlled by TORC2, including actin polymerization, endocytosis, and sphingolipid synthesis (Cybulski and Hall, 2009; Bartlett and Kim, 2014) are also defective in the Δ*gup1* mutant (Ni and Snyder, 2001; Casamayor and Snyder, 2002; Ferreira and Lucas, 2008).

Pil1 has a cell cycle-regulated expression, which is consistent with eisosome assembly in growing buds (Moreira *et al.*, 2009). In fact, eisosomes are formed *de novo* in the bud of dividing cells in a polarized wave manner from the neck to the tip of bud (Moreira *et al.*, 2009). As the bud grows, an approximately even distribution of eisosomes is reached, indicating a tightly controlled process linked to plasma membrane expansion (Moreira *et al.*, 2009). Interestingly, Pil1 expression is cell cycle-regulated (Spellman *et al.*, 1998), and occurs in 20 min bursts that correlate with the initiation of eisosome formation as well as with membrane expansion during cell cycle progression, thus synchronizing eisosome formation with plasma membrane growth (Moreira *et al.*, 2009). This away, cell cycle regulation of Pil1 levels is crucial to eisosome biogenesis.

In the past few years the description of proteins linked to eisosomes has steadily increased (Grossmann *et al.*, 2008; Deng *et al.*, 2009; Fröhlich *et al.*, 2009; Aguilar *et al.*, 2010), although Gup1 was never identified as one of them. Concomitantly, the sub-cellular localization of Gup1 in this furrow-like structure was never observed. Instead, this protein exhibits a homogeneous distribution over the plasma membrane (Hölst *et al.*, 2000; Bleve *et al.*, 2005). This could imply that the interaction between Gup1 and Pil1 described in this work is either transient or it could happen in other membrane or ER locations, prior to, or even after, eisosomes assembly. This chapter presents a first assessment of the putative phenotypes derived from the interaction between Gup1 and Pil1. It was verified that the number of eisosomes is reduced in the absence of Gup1 though the expression of Pil1 was not affected. Moreover, the double absence of Gup1 and Pil1 induces an increased susceptibility SDS, a membrane associated stress, which suggests that the interaction between both proteins is important to membrane composition, integrity and/or stability.

Material and Methods

Strains and growth conditions

The bacteria and yeast strains used in this study are listed in Table 1. *Escherichia coli* XL1 Blue was purchased from Stratagene. Bacteria were cultivated in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, (2% agar for solid medium) pH 7.2 appropriately supplemented for antibiotic resistance when necessary (100 mg/mL ampicillin or 50 µg/mL kanamycin). Cultivation of bacteria, as well as isolation and manipulation of plasmid DNA, were done using standard procedures (Ausubel *et al.*,1999).

Yeast were cultivated on YPD (1% yeast extract, 2% peptone, 2% glucose, 2 (2% agar for solid medium)), or YNB medium (0.175% YNB without amino acids and nitrogen source (Difco), 0.5% $(NH_4)_2SO_4$, 2% glucose or galactose) appropriately supplemented according to auxotrophic requirements. Liquid cultures were performed in batch at 30 ºC and 200 rpm orbital shaking in a 1/3 air to liquid ratio.

Table 1 - Microbial strains used in the present study.

Strain	Genotype	Source
S. cerevisiae BY4741 *wt*	*MATa; ura3Δ0; leu2Δ0; his3Δ1; met15Δ0*	Euroscarf
S. cerevisiae BY4741 Δ*gup1*	*MATa; ura3Δ0; leu2Δ0; his3Δ1; met15Δ0; YGL084c::kanMX4*	Euroscarf
S. cerevisiae BY4741 Δ*pil1*	*MATa; ura3Δ0; leu2Δ0; his3Δ1; met15Δ0; YGR086C::kanMX4*	Euroscarf
S. cerevisiae BY4741 Δ*gup1*Δ*pil1*	*MATa; ura3Δ0; leu2Δ0; met15Δ0; YGR086C::kanMX4; YGL084c::HIS3*	This study
S. cerevisiae BY4741 Δ*pil1 - PIL1-GFP*	*MATa; ura3Δ0; leu2Δ0; met15Δ0; YGR086C::PIL1-GFP-HIS3*	Huh *et al.*, 2003
S. cerevisiae BY4741 Δ*gup1*Δ*pil1 - PIL1-GFP*	*MATa; ura3Δ0; leu2Δ0; his3Δ1; met15Δ0; YGR086C::PIL1-GFP-HIS3; YGL084c::kanMX4*	This study
E. coli XL1 Blue	*endA1gyrA96 (nalR) thi-1 recA1 lac glnV44 F'[::Tn10 proAB+ lacIq Δ(lacZ)M15]hsdR17(rK- mK+)*	Stratagene

Construction of *S. cerevisiae* Δ*gup1*Δ*pil1* double mutant and Δ*gup1*Δ*pil1-PIL1-GFP*

The double mutant of *S. cerevisiae* Δ*gup1*Δ*pil1* was constructed replacing the *GUP1* gene in BY4741 Δ*pil1* (Euroscarf) with the *gup1::HIS3* disruption cassette amplified by PCR from the p416 plasmid (Addgene), using primers A and B (Table 2). The *gup1::HIS3* disruption cassette was used to transform BY4741 Δ*pil1* strain by homologous recombination using standard protocols (Ito *et al.*, 1983). The generated transformants were selected in YNB medium without histidine. Positive clones were confirmed by colony PCR using the *GUP1* deletion confirmation primers E and F (Table 2). The construction of the Δ*gup1*Δ*pil1-PIL1-GFP* was performed by deleting the *GUP1* gene from the Δ*pil1-PIL1-GFP* strain (kindly provided by Erin K. O'Shea, Howard Hughes Medical Institute (Huh *et al.*, 2003)). The *GUP1* gene was deleted using the KanMx disruption cassette, amplified from pUG6 plasmid (Addgene), using primers C and D listed in Table 2. The *gup1::KanMx* disruption cassette was used to transform Δ*pil1-PIL1-GFP* strain by homologous recombination using standard protocols (Ito *et al.*, 1983). Transformants were selected in YNB medium with geneticin (200mg/L). Positive clones were confirmed by colony PCR using *GUP1* deletion confirmation primers E and F, listed in the Table 2.

Table 2 - Primers used in the present study and their sequence.

Name	Primer
A - Fw *gup1::HIS3* cassette	5'ATGTCGCTGATCAGCATCCTGTCTCCCCTAATTACTTCCGTTTC CCGCAATTTTCTTTTTC 3'
B - Rv *gup1::HIS3* cassette	5'TCAGCATTTTAGGTAAATTCCGTGCCTCTTTTCTTCTTCTATAT ATATCGTATGCTGCAGC 3'
C - Fw *gup1::KanMx* cassette	5'ATGTCGCTGATCAGCATCCTGTCTCCCCTAATTACTTCCGGAC ATGGAGGCCCAGAATAC 3'
D - Rv *gup1::KanMx* cassette	5'TCAGCATTTTAGGTAAATTCCGTGCCTCTTTTCTTCTTCTCAGT ATAGCGACCAGCATTC 3'
E - Fw *GUP1* deletion confirmation	5' ATCAGCTCAATCGGACATA 3'
F - Rv *GUP1* deletion confirmation	5' ATCATATGGTCCAGAAACC 3'
G - Rv *GUP1* deletion confirmation	5' CTGCAGCGAGGAGCCGTAAT 3'

Total RNA isolation

Yeast samples for real-time PCR analysis (~$5x10^7$ cells) were collected and the cell pellets were mechanically disrupted using 0.5 mm ø glass beads in a swing-mill at 30 Hz for 15 min. Total RNA was extracted and isolated using the NucleoSpin® RNA kit (Macherey-Nagel), and subsequently quantified in a ND-1000 UV-visible light spectrophotometer (NanoDrop Technologies). RNA quality was evaluated by agarose-gel electrophoresis. The absence of contaminant gDNA was verified by directly using the isolated RNA as template in real-time PCR assays (*i.e.* RNA not reverse-transcribed to cDNA).

Quantitative Real Time-PCR (qRT-PCR)

Primers for qRT-PCR (Table 3) were built using Primer3Plus software, aligned against *S. cerevisiae* genome sequence (www.yeastgenome.org/blast-sgd) for specificity confidence, and analysed with the Mfold server (http:/unafold.rna.albany.edu/?q=mfold) to check for the possible formation of self-folding secondary structures. Total RNA (500 µg) was reverse-transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad). The cDNA levels were then analysed using the Bio-Rad® CFX96 Touch™ real-time PCR instrument. Each sample was tested in duplicate in a 96-well plate (Bio-Rad, CA). The reaction mix (10 µL final volume) consisted of 5 µL of SsoAdvanced™

SYBR® Green Supermix (Bio-Rad), 0.25 μL of each primer (250 nM final concentration) and 1 μL of cDNA preparation. A blank (no template) control was included in each assay. The thermocycling program consisted of one hold at 98°C for 30 sec, followed by 40 cycles of 10 sec at 98°C and 20 sec at 60°C. After completion of these cycles, a melting-curve was performed (65°C-95°C; 0.5°C increments, 3s) and data collected to verify PCR specificity, contamination and the absence of primer dimers. Three different extractions of total RNA were analysed, by at least duplicate PCRs. The data were normalized to *18S* gene. The comparative Ct method analysis ($2^{-\Delta\Delta CT}$ method) (Schmittgen and Livak, 2008) was used to analyse the results. At least three different RNA extractions were performed and analysed.

Table 3 - qRT-PCR primers used in the present study and their sequence.

Name	**Primer**
Fw *GUP1* qRT-PCR	5' GCGTGGGAAAATGACACAC 3'
Rv *GUP1* qRT-PCR	5' AAACAGCCTCCACGGAATC 3'
Fw *PIL1* qRT-PCR	5' GCGCACTGAATGAATGGAC 3'
Rv *PIL1* qRT-PCR	5' TTGTTCGTCTTCGGACCAC 3'
Fw *18S* qRT-PCR	5' TGCGATAACGAACGAGACC 3'
Rv *18S* qRT-PCR	5' TCAAACTTCCATCGGCTTG 3'

Microscopy procedures

Fluorescence microscopy. For sub-cellular localization of Pil1-GFP and quantification of eisosomes, cells were collected from mid-exponential yeast cultures grown in YNB glucose. Cells were then observed in a Leica Microsystems DM-5000B epifluorescence microscope with the appropriate filter settings, using a 100x oil-immersion objective. Images were acquired through a Leica DCF350FX digital camera

and processed with LAS AF Leica Microsystems software. At least 300 cells were counted per experiment.

Light microscopy. Cellular morphology was observed by light microscopy in cells collected from mid-exponential yeast cultures. Microscopy assessments were done in a Leica Microsystems DM-5000B epifluorescence microscope. Images were acquired through a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software. To observe colony morphology/differentiation, cells from mid-exponential yeast cultures were diluted 100x, spotted (50 µL) on YPD (1% glucose), and incubated for 12 days at room temperature. Resulting colonies were visualized in a Leica Zoom 2000 stereo microscope and the images were acquired through a Leica EC3 digital camera and processed with LAS AF Leica Microsystems software.

Sedimentation and stress phenotypes

Culture sedimentation. To assess the sedimentation phenotype, cells were grown to mid-exponential phase in YPD, at 30ºC. Cultures were collected to a final $O.D._{600}$=1 in a micro-tube and left to rest at room temperature for 20 min, after which the resultant sedimentation was photographed.

Drop tests. Drop tests were performed using cell suspensions at a final $O.D._{600}$=1, collected from mid-exponential YPD grown cultures. Four 10-fold serial dilutions were made, and 5 µL of each suspension was applied on respective medium. Results were scored after 3 days of incubation at 30ºC, unless otherwise stated.

Mat formation

The ability of yeast strains to form a mat was assessed as previously described (Reynolds and Fink, 2001), with some modifications. Overnight cultures were collected by centrifugation, diluted to a final $O.D._{600nm}$=1 in water and 5 µL of this suspension was used to inoculate 0.3% agar YPD plates (all plates were prepared at the same time (one day before) to ensure the same level of medium hydration). The plates were then sealed with parafilm and incubated at room temperature. Results were scored after 12 days of incubation by measuring the diameter of the mat.

Effect of acetic acid on yeast viability

Yeast strains were grown until mid-exponential phase ($O.D._{600}$ = 0.6–0.8) on YNB medium, after which they were collected and resuspended to a final $O.D._{600}$ = 0.2 in fresh YNB adjusted to pH 3.0 with HCl and containing 150 mM acetic acid. Incubation took place for 180 min at 30°C. At determined time points, 40 µL 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 the number of c.f.u. obtained at T_0.

Quantification of PI staining by flow cytometry

Flow cytometry was used to assess membrane integrity by counting the cells stained with propidium iodide (PI) (Sigma Aldrich). Cells were harvested, washed and resuspended in PBS containing 4 µg/mL PI. The samples were incubated for 10 min at room temperature, in the dark, and analysed in an Epics® XL™ (Beckman Coulter) flow cytometer. At least 20,000 cells from each sample were analysed.

Results and Discussion

PIL1 expression and localization in the Δ*gup1* mutant

Pil1, as mentioned above, was one of the two novel proteins identified as physical partners of Gup1 (Chpt. 2). This protein is a highly abundant constituent of the structural core of eisosomes (Zhang *et al.*, 2004; Walther *et al.*, 2006). Taking that into account, the expression of *PIL1* in Δ*gup1* mutant was assessed by qRT-PCR, quantified relative to the ribosomal subunit *18S*, and compared with the relative expression level in the corresponding *wt* strain. *PIL1* expression in the Δ*gup1* mutant was comparable to that observed in *wt* strain (Fig. 1A), suggesting that the expression of *PIL1* is not affected by the absence of Gup1. Subsequently, the sub-cellular localization of Pil1 in the Δ*gup1* mutant was also assessed in comparison to *wt*, using the strains expressing the chimeric Pil1-GFP (Fig. 1B). Pil1 is a peripherical-membrane protein that is distributed in punctate patches corresponding to the eisosomes (Moreira *et al.*, 2009;

Strádalová *et al.*, 2009). This kind of distribution was kept in the Δ*gup1* mutant cells, identical to *wt*, as showed in the Fig. 1B. However, when Gup1 is not present, the number of punctate structures (corresponding to the eisosomes) was significantly reduced (Fig. 1C). The number of eisosomes per cell was quantified, and shown to decrease from approximately 40 in *wt* cells to approximately 20 in the Δ*gup1* mutant strains (Fig. 1C).

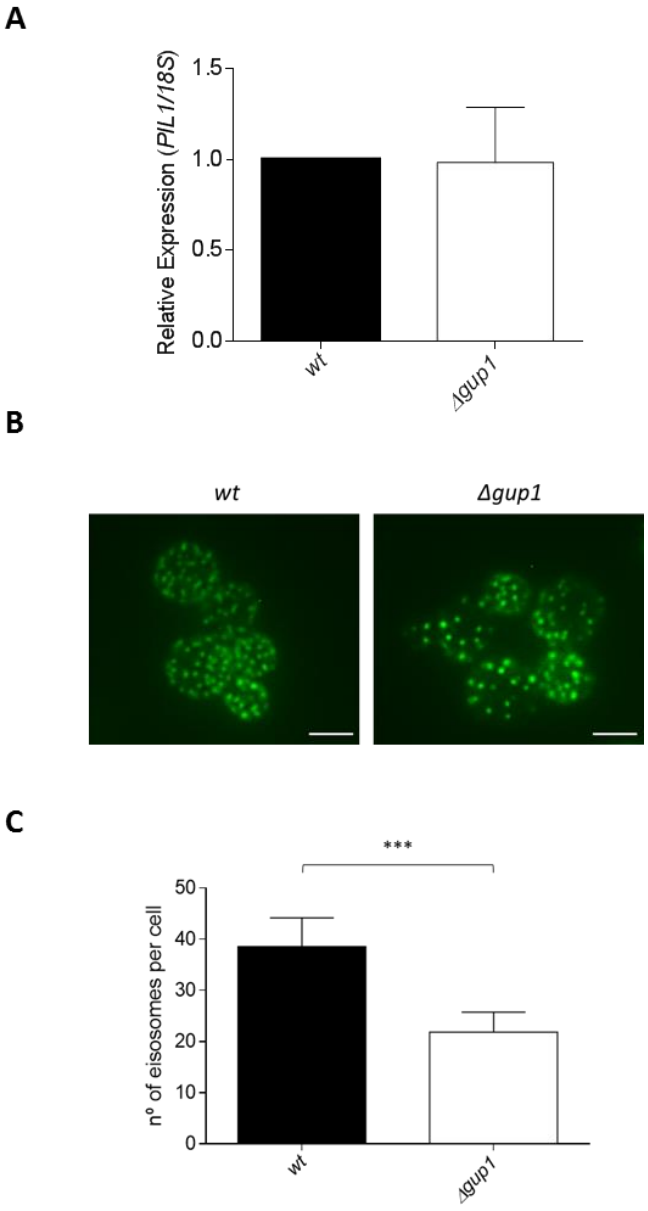

Figure 1 - *PIL1* expression (A), localization (B) and number of eisosome (C) in *S. cerevisiae* BY4741 *wt* and *Δgup1* strains. Cells were grown in YNB glucose medium until mid-exponential phase. **(A)** Relative expression of *PIL1* by qRT-PCR. *PIL1* expression was normalized against *18S* and represented relative to the levels in the *wt* strain as calculated by the comparative Ct method analysis ($2^{-\Delta\Delta CT}$ method). **(B)** The Pil1 localization was assessed using the *wt* and Δ*gup1* strains with a chromosomal Pil1-GFP insertion. **(C)** The number of eisosomes structures were counted in 300 cells per experiment. Results are representative of at least three independent experiments. ***P < 0.001 t-test.

A smaller number of normal-sized eisosomes, or a normal number of larger eisosomes, was described for strains with reduced or increase levels of Pil1, respectively (Moreira *et al.*, 2009). However, in the present case, the Δ*gup1* mutant strain did not present any significant change in Pil1 expression that could justify the reduced number of eisosomes. Therefore, other mechanisms limiting eisosome formation must be affected. Membrane association of Pil1 seems to be the limiting step in eisosome formation (Moreira *et al.*, 2009; Olivera-Couto *et al.*, 2011). The deletion of *GUP1* causes profound alteration in lipids composition (Oelkers *et al.*, 2000; Bosson *et al.*, 2006) and organization (Bosson *et al.*, 2006; Ferreira and Lucas, 2008), the implications of which in eisosome structure and number were never assessed before.

The deletion of *PIL1* results in miss localization of all tested MCC/eisosome components (Walther *et al.*, 2006; Grossmann *et al.*, 2007, 2008). For example, a few big clusters of Lsp1, designated "eisosome remnants", were formed in the plasma membrane of the Δ*pil1* cells (Walther *et al.*, 2006). The sterols usually present in the MCC/eisosomes also lose their characteristic punctate pattern in the Δ*pil1*, and spread through the plasma membrane, some concentrating at eisosomes remnants (Walther *et al.*, 2006; Grossmann *et al.*, 2007, 2008). An identical disorganization on ergosterol distribution in the plasma membrane was previously described in Δ*gup1* cells (Ferreira and Lucas, 2008). In fact, deep alterations on the integrity and assembly of the sphingolipid-sterol ordered domains were related to the absence of *GUP1*, as evidenced by (i) the homogeneous distribution of sterols in the Δ*gup1* mutant plasma membrane, (ii) the 40% decrease in Detergent Resistant Membrane (DRM) domains recovered, and (iii) the reduced quantities of some proteins associated with these lipid micro-domains, like the GPI-anchored Gas1 and the H^+-ATPase Pma1 (Ferreira and Lucas, 2008). Deletion of *GUP1* also causes changes in the regular concentrations of major types of lipids: a high increase in diacyl- and triacylglycerol, a decrease in phospholipids, and an accumulation of lyso-PI (Oelkers *et al.*, 2000; Bosson *et al.*, 2006). Therefore, the reduction of eisosome structures in this mutant could be a consequence of the incorrect ergosterol distribution and membrane composition. Nevertheless, the opposite hypothesis cannot be ruled out. The reduction of eisosomes could provoke the alteration on ergosterol distribution observed in Δ*gup1* mutant. The way Gup1 affects eisosome formation, and if it is a direct effect on Pil1 assembly, requires further investigation. In

particular, Pil1 is regulated by Pkh1 and Pkh2 kinases phosphorylation, though it is controversial whether this promotes assembly or disassembly of eisosome domains (Walther *et al.*, 2006; Luo *et al.*, 2008; Deng *et al.*, 2009). The phosphorylation state of Pil1 in the absence of the Gup1 would thus be interesting to evaluate in the future. Moreover, the possibility that Gup1, as an acyl-transferase, could operate some lipid modification of Pil1, this way interfering with the protein ability to associate with the membrane, cannot be discarded.

Pil1 binds preferentially to plasma membrane PI(4,5)P2 (Karotki *et al.*, 2011; Fröhlich *et al.*, 2014). In fact, it was shown that the mutation of the Pil1 residues that interact with PI(4,5)P2 diminishes the ability of Pil1 to bind membranes, and decreases the number of eisosomes that are formed (Olivera-Couto *et al.*, 2011). Moreover, reduction of PI(4,5)P2 also causes a decrease in the total number of eisosomes (Karotki *et al.*, 2011). The absence of enzymes that catalyse the last steps of PI(4,5)P2 synthesis (Pik1, Stt4, Sac2, Mss4) produces phenotypes that are common to some of those exhibited by Δ*gup1* mutant (Lucas *et al.*, 2016). These include, for instance: (i) the reduced autophagy and abnormal vacuole morphology of Δ*pik1* mutant (Audhya *et al.*, 2000; Wang *et al.*, 2012), (ii) the involvement of the Stt4 on cell wall integrity, and actin cytoskeleton organization (Yoshida *et al.*, 1994; Audhya *et al.*, 2000), (iii) the Δ*sac1* abnormal protein trafficking and sphingolipid biosynthesis (Schorr *et al.*, 2001; Brice *et al.*, 2009), and (iv) the relation between the Mss4 with several morphogenic processes (Homma *et al.*, 1998). It is, therefore, reasonable to consider the possibility of Gup1 acting upstream of these enzymes, at the level of phosphoinositol (PI) production. A change in PI availability would provide a reason for the changes observed at the level of eisosomes formation in Δ*gup1* cells. It could also explain the increased levels of diacyl- and triacylglycerols present in this mutant (Oelkers *et al.*, 2000).

In addition, several genetic screens identified genes involved in various aspects of lipid synthesis that are needed for proper eisosome formation and/or stability (Grossmann *et al.*, 2008; Fröhlich *et al.*, 2009; Strádalová *et al.*, 2009; Aguilar *et al.*, 2010). Elevated sphingolipids stimulate Nce102 to localize to MCCs, where it promotes eisosome formation by inhibiting the phosphorylation of Pil1 and Lsp1 by the above-mentioned Pkh1/2 kinases (Fröhlich *et al.*, 2009). Sphingolipid levels also affect the eisosomal localization of Slm1 and Slm2 proteins, which are also involved in the formation of eisosomes and associated signalling through TORC2 (Kamble *et al.*, 2011;

Olivera-Couto *et al.*, 2011; Berchtold *et al.*, 2012). Several genes involved in ergosterol synthesis were also identified as essential for eisosome formation and/or stability (Swain *et al.*, 2002; Grossmann *et al.*, 2008), however, because impaired ergosterol synthesis causes a corresponding decrease in sphingolipids, it is not clear if ergosterol plays a direct role in the formation of these structures (Swain *et al.*, 2002; Guan *et al.*, 2009). The interference of Gup1 in sphingolipid biosynthesis, evidenced by the decreased resistance of the mutant to inhibitors of specific biosynthetic steps (Ferreira and Lucas, 2008), will be discussed further ahead.

Effects of Pil1 and its interaction with Gup1

Cell membrane and wall stress-related phenotypes

The *wt*, Δ*gup1*, Δ*pil1* and the double mutant Δ*gup1*Δ*pil1* were used to better assess the biological functions associated to the Gup1 and Pil1 interaction, and putative associated processes. The deletion of *GUP1* gene, as above mentioned, affects lipid metabolism (Ferreira and Lucas, 2008), and the integrity/biogenesis of the plasma membrane, namely at the level of sphingolipid-sterol ordered domains (lipid rafts) (Ferreira and Lucas, 2008). Moreover, the deletion of *GUP1* gene, also causes a severe modification in the cell wall composition and structure (Ferreira *et al.*, 2006) compatible with the (i) increased sensitivity to high temperature, (ii) sensibility to several cell wall disturbing drugs, and (iii) the aggregation phenotype, exhibited by Δ*gup1* mutant. Accordingly, as a consequence of the alterations on cell wall composition, the Δ*gup1* mutant was previously reported to form large and heavy cell aggregates that swiftly sediment (Ferreira *et al.*, 2006). The aggregation and sedimentation phenotypes of Δ*gup1* mutant (Fig. 2) were not observed in either *wt* or Δ*pil1* cells, but prevailed in the Δ*gup1*Δ*pil1* double mutant (Fig. 2). This suggests that Pil1 as well as Gup1-Pil1 interaction do not implicate on yeast aggregation/sedimentation, and it also suggests that the cell wall of the Δ*pil1* should be unaltered, similarly to *wt*.

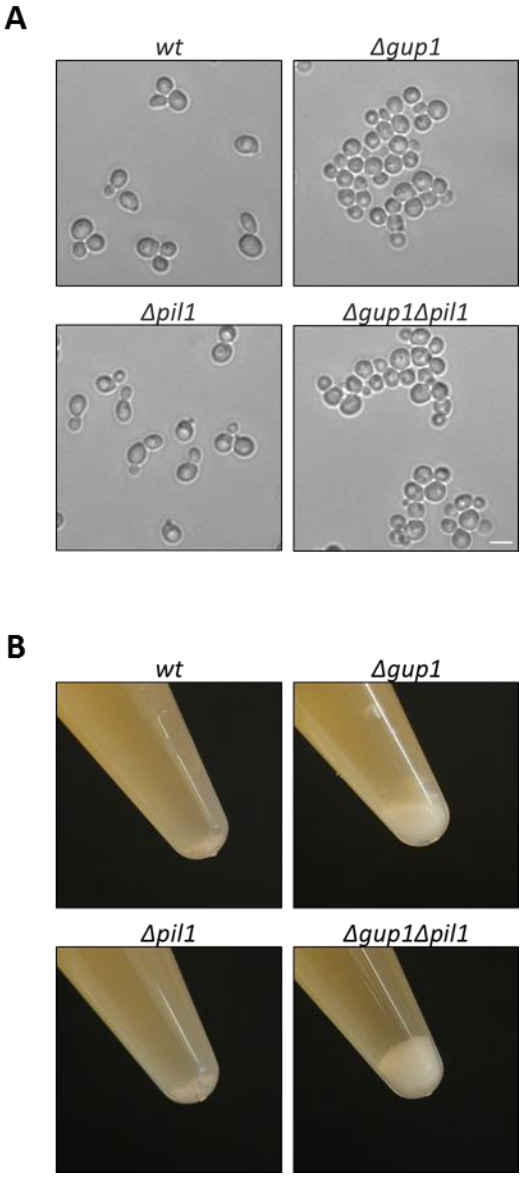

Figure 2 – Cell aggregation and sedimentation of *S. cerevisiae* BY4741 *wt, Δgup1, Δpil1 and Δgup1Δpil1* strains. The assays were performed in mid-exponential cells grown in YPD. **(A)** Cell aggregation was visualized by light microscopy. **(B)** Sedimentation phenotype was recorded after letting the culture rest at room temperature for 20 min. One representative experiment is shown.

To test whether Pil1 introduced any changes in what regards cell wall, and if the interaction of Pil1 with Gup1 is important for the associated roles/phenotypes, the single and double deleted strains were exposed to well-known wall disruption agents. Directly disrupting the cell wall integrity are Congo Red (CR) and Calcofluor White (CFW)

(Ram and Klis, 2006), which bind to chitin that way interfering with proper cell wall assembly (Roncero and Durán, 1985), and caffeine that indirectly activates the CWI pathway through the TORC1 protein kinase complex (Lum *et al.*, 2004; Kuranda *et al.*, 2006). Indirectly, the construction of the cell wall is also known to be affected by SDS detergent (Igual *et al.*, 1996). Cells were exposed, as previously described, to these agents and their viability quantified by drop tests (Fig. 3 – left panels). Additionally, cells were further incubated with 1 M sorbitol, which is known to remediate growth defects associated with cell wall instability by osmotically stabilizing the damaged cells (Levin, 2005) (Fig. 3 – right panels). The results observed with the Δ*gup1* mutant were all as expected (Ferreira *et al.*, 2006; Ferreira and Lucas, 2008), consistent with a primary defect in cell wall biogenesis and composition. On the other hand, the deletion of *PIL1* did not provoke any significant phenotypes in the presence of CR, CFW or caffeine (Fig. 3 – left panels), consistently with the absence of aggregation/sedimentation above mentioned. As mentioned before, despite these defects, the CWI pathway is functioning properly in the Δ*gup1* mutant (Ferreira *et al.*, 2006), Therefore, the susceptibility of Gup1 to cell wall perturbing agents should result from malfunction of other signalling or metabolic pathways. Two other pathways contribute to the wall integrity and remodelling: the Sho1 branch of the HOG pathway, and the sphingolipids long chain bases (LCB) TORC2/Ypk1 pathway mentioned above (Lagorce *et al.*, 2003; Boorsma *et al.*, 2004; García *et al.*, 2004). SDS indirectly affects the cell wall, as mentioned above (Igual *et al.*, 1996), but as a detergent it affects the plasma membrane directly. In opposition to CFW and CR, the Δ*pil1* mutant was more sensitive to SDS than the Δ*gup1* (Fig. 3 – left panels), a phenotype that was not reversed by the inclusion of 1 M sorbitol in the growth medium (Fig. 3 – right panels). This result is in line with the role of Pil1 on eisosome formation in the plasma membrane (Moreira *et al.*, 2009), affecting its stability but not affecting the wall, not even indirectly. On the other hand, the double mutant Δ*gup1*Δ*pil1* was generally more sensitive to all treatments, including SDS. The enhanced susceptibility of Δ*gup1*Δ*pil1* to the wall-perturbing agents, in spite that no phenotype was observed in the Δ*pil1* single mutant, might be an indirect effect deriving from an increased disruption of the membrane as evidenced from the increased sensitivity to SDS.

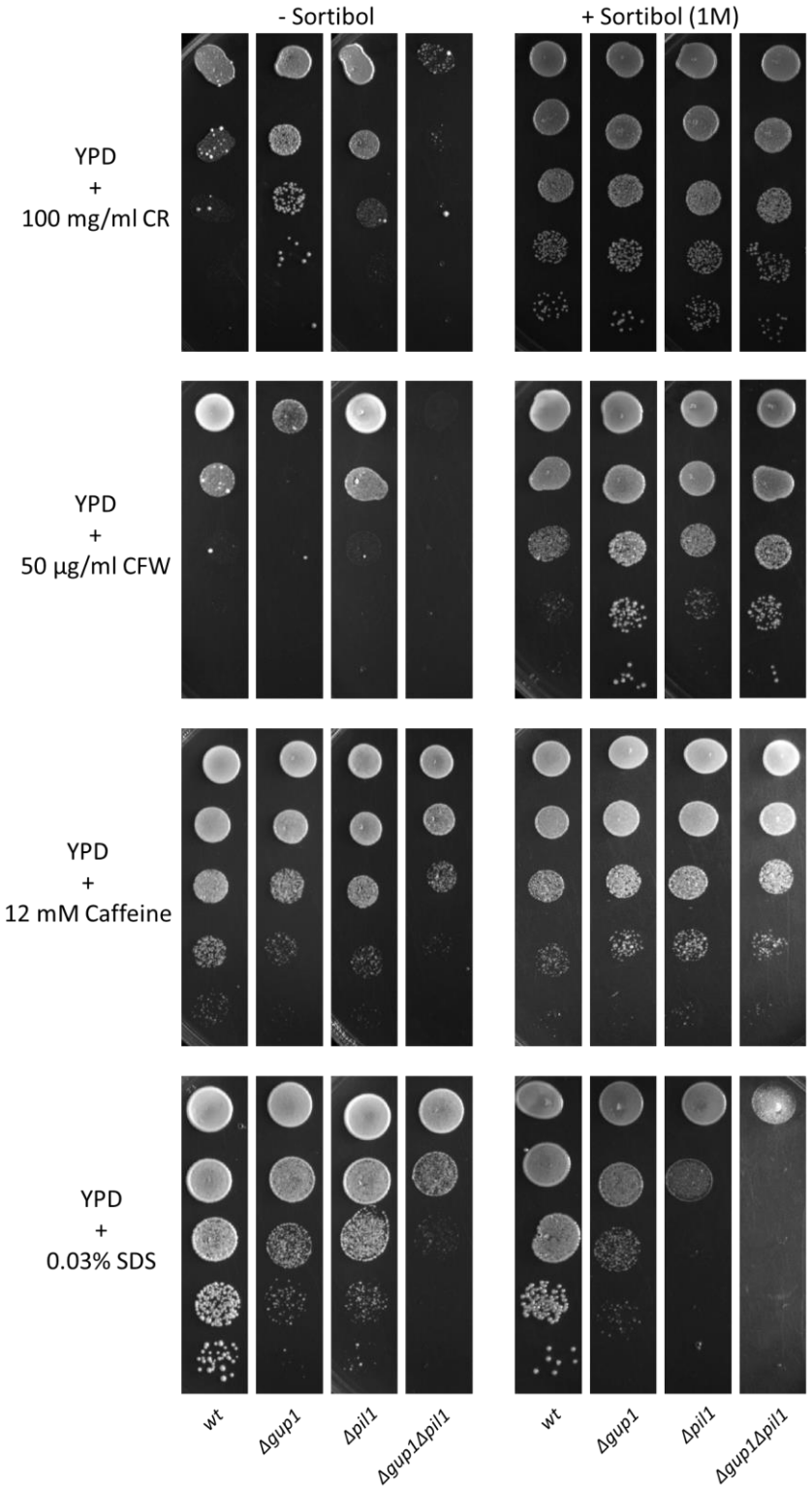

Figure 3 – Membrane and cell wall stress-related phenotypes of *S. cerevisiae* BY4741 *wt, Δgup1, Δpil1* and *Δgup1Δpil1* strains. Cells were grown on YPD until O.D.$_{600}$=1 and 10-fold serial dilutions of each culture were spotted on different media. Results were scored after a 3 days incubation at 30˚C. One representative experiment is shown.

The growth of *S. cerevisiae wt, Δgup1, Δpil1* and *Δgup1Δpil1* was assessed at 30ºC and 37ºC, using different carbon sources (Fig. 4). The Δ*gup1* single mutant was previously described to exhibit decreased growth on non-fermentable carbon sources, a phenotype that is aggravated at 37 ºC (Ferreira *et al.*, 2006). Additionally, a small decrease in growth ability at 37ºC was also reported for Δ*gup1* cells growing on YPD medium (Ferreira *et al.*, 2006). In the present work, however, no significant decrease was observed in the growth ability of Δ*gup1* mutant in YPD medium at 37ºC, and only a small decrease when grown in glycerol (Fig. 4). It should be noted, nevertheless, that the Δ*gup1* yeast strains used in this work is from a different genomic background than those used in previous works (Ferreira *et al.*, 2006). The results observed in this work could be more easily correlated to a role of mitochondria, rather than major defects on the cell wall. Still, the results obtained in this study, regarding sensitivity to CFW and caffeine (Fig. 3), as well as the aggregation/sedimentation phenotype (Fig. 2), of Δ*gup1* all indicate the existence of cell wall-related defects in these cells.

The sensitivity of Δ*gup1*, described by Ferreira and colleagues, was suggested to result from major defects on the cell wall caused by *GUP1* deletion (Ferreira *et al.*, 2006), but it could also derive from the altered sphingolipid metabolism caused by the absence of Gup1 (Ferreira and Lucas, 2008). Sphingolipids are structural components of membranes that have important signalling functions, being central regulators of many cellular processes (for a review see Montefusco *et al.*, 2014) including the response to heat stress (Dickson *et al.*, 1997; Jenkins *et al.*, 1997). Heat stress provokes an increase in the amounts of sphingolipids LCB in the membrane (Dickson *et al.*, 1997; Jenkins *et al.*, 1997), which stimulate the activation of the Ypk1, Sch9 and CWI/PKC pathways through phosphorylation by Pkh1/2 kinases (Inagaki *et al.*, 1999; Roelants *et al.*, 2002; Swinnen *et al.*, 2014), the same kinases that phosphorylate Pil1, possibly interfering in eisosome formation as discussed above. These pathways work in parallel to control the cellular processes associated with stress response, namely the regulation of cell wall integrity and repolarization of the actin cytoskeleton during heat stress (Delley and Hall, 1999; Levin, 2011). Importantly, Gup1 is implicated in several lipid-associated phenotypes. The lipid membrane composition of *Δgup1* cells in comparison to *wt* presents a reduced fraction of phospholipids and elevated levels of triacylglycerols and diacylglycerols (Oelkers *et al.*, 2000). Gup1 also interferes in sphingolipid biosynthesis, as evidenced by the decreased resistance of the mutant to inhibitors of specific

biosynthetic steps (Ferreira and Lucas, 2008). An increased susceptibility to such inhibitors is usually taken as indicative of an altered lipid metabolism (Georgopapadakou and Walsh, 1996). Altogether, besides major cell wall defects, the altered lipid metabolism and membrane composition could be responsible for the sensitivity of Δ*gup1* to high temperatures (Ferreira *et al.*, 2006), suggesting a regulatory role of the Gup1 protein on the Ypk1 or Sch9 pathway. The CWI pathway must be excluded in view of the regular dual phosphorylated state of Slt2 after stimulation of the pathway (Ferreira *et al.*, 2006). Accordingly, Δ*gup1* was found to display profoundly aberrant random bud site selection, and a large defect on the proper establishment of actin cytoskeleton polarity (Ni and Snyder, 2001; Casamayor and Snyder, 2002), which are common consequences of the malfunction of the above-mentioned pathways (Schmidt *et al.*, 1996; Kamada *et al.*, 2005).

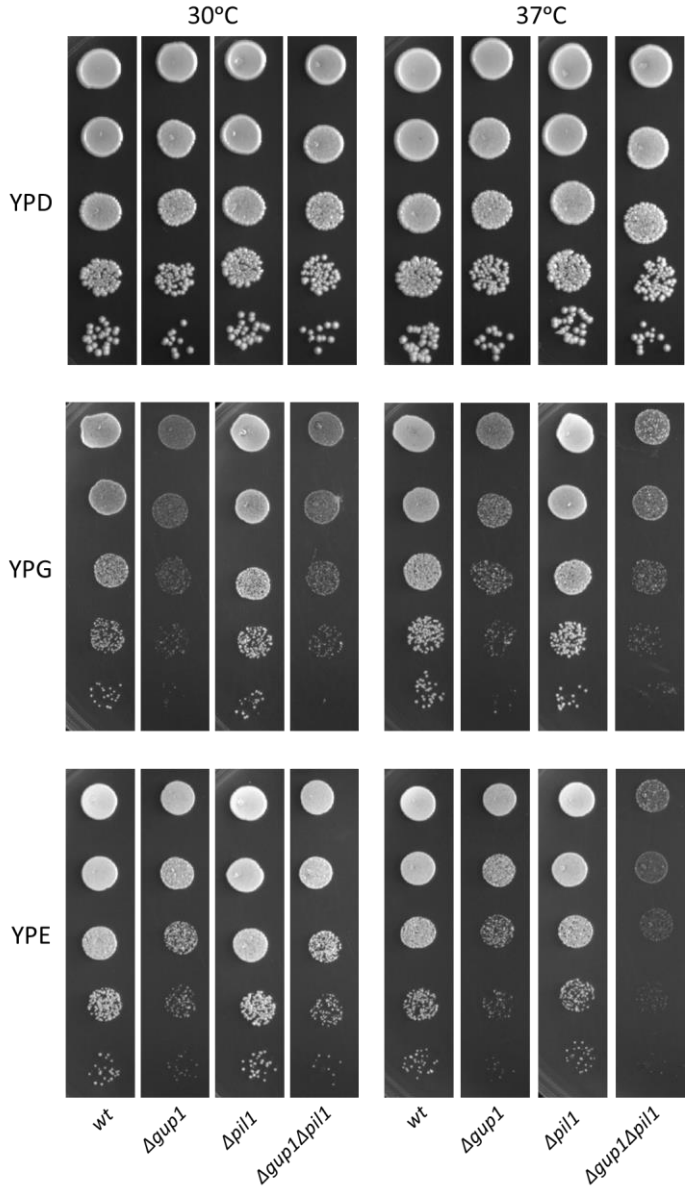

Figure 4 – Carbon source and high temperature-related phenotypes of *S. cerevisiae* BY4741 *wt*, *Δgup1*, *Δpil1* and *Δgup1Δpil1* strains. Strains were grown on YPD until $O.D._{600}$=1 and 10-fold serial dilutions of each culture were spotted on YP with 2% (w/v) glucose (YPD), glycerol (YPG) or ethanol (YPE). Results were scored after 3 days incubation at 30˚C or 37 ˚C. One representative experiment is shown.

On the other hand, Pil1 has been pointed as a negative regulator of heat stress resistance, being the mutant Δ*pil1* more resistant to high temperatures (Zhang *et al.*, 2004). In the present work, Δ*pil1* cells behaved similarly to *wt* at 37ºC, and no resistance phenotype was perceptive (Fig. 4). It should be mentioned, however, that the resistance to high temperatures observed by Zhang and colleagues (2004) was determined by c.f.u. counting, while the drop tests performed in this work were not sensitive enough to discriminate such effect. Pil1 regulation, as mentioned above, depends on phosphorylation by Pkh1/2 (Zhang *et al.*, 2004; Walther *et al.*, 2006). Zhang and colleagues (2004) propose that upon heat stress, the transient increase in LCB acts to inhibit Pil1 phosphorylation by Pkh1/2 protein kinases. Non-phosphorylated Pil1 frees Pkh1/2, allowing these proteins to phosphorylate and activate CWI/PKC, Sch9 and Ypk1 pathways (Jenkins *et al.*, 1997; Zhang *et al.*, 2004). When both *GUP1* and *PIL1* are absent, the resultant double mutant became less thermotolerant than Δ*pil1*, behaving identically to Δ*gup1* mutant. The exception occurs when using ethanol as carbon source at 37ºC, in which case the double mutant was even more sensitive (Fig. 4). These results suggest that Gup1 and Pil1 proteins are players in the same pathways, possibly those related to Sch9 and Ypk1 signalling, and that Gup1 is important for the down-regulation exerted by Pil1 in the heat stress response.

Gup1 was long thought to be important for high osmotic stress survival (Hölst *et al.*, 2000; Ferreira *et al.*, 2006). Moreover, its sensitivity to CFW despite the operationality of the CWI pathway, could indicate, as above said, a malfunction of the HOG pathway. Consequently, the single and double mutants were cultivated in the presence of 1 M NaCl (Fig. 5 – upper panel) or 1.5 M KCl (Fig. 5 – lower panel) to induce the HOG pathway activation. All the Δ*gup1*, Δ*pil1* and Δ*gup1/*Δ*por1* mutants were equally more sensitive to osmotic stress than *wt* (Fig. 5). A decreased growth under high osmotic stress was already described for the Δ*gup1* mutant (Hölst *et al.*, 2000; Ferreira *et al.*, 2006). This indicates a malfunction of the HOG pathway in this mutant and a concomitant deficient production and/or accumulation of glycerol (Hölst *et al.*, 2000).

The pronounced phenotype of Δ*gup1* mutant in the presence of CFW (Fig. 3) that triggers both HOG and CWI/PKC pathways (Bermejo *et al.*, 2008), compared with the minor effect caused by CR (Fig. 3) that triggers only CWI/PKC (Kuranda *et al.*, 2006), also points to the malfunction of HOG. Pil1 protein is up regulated under high osmotic stress (Szopinska *et al.*, 2011), which concurs with the sensitive behaviour of the Δ*pil1* mutant in the presence of NaCl and KCl (Fig. 5). Moreover, MCC/eisosome domains have also been suggested to be involved in the response to hyperosmotic conditions (Dupont *et al.*, 2010), functioning as backup reservoirs of membrane for stretching needs in the face of osmotic unbalance (Kabeche *et al.*, 2015a).

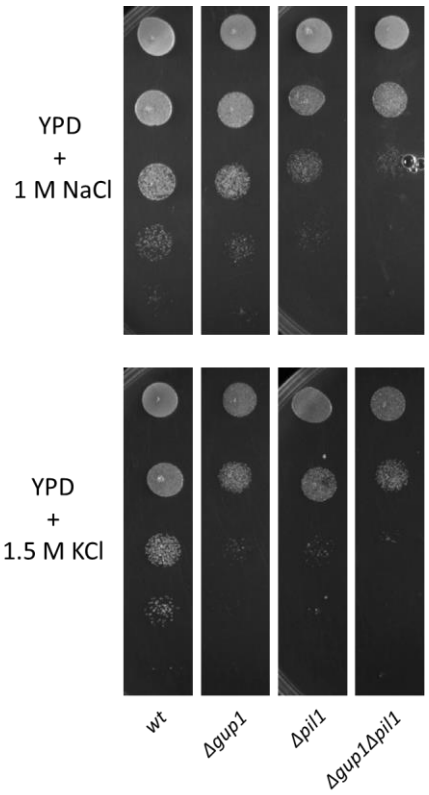

Figure 5 – Osmotic stress phenotypes of *S. cerevisiae* BY4741 *wt*, *Δgup1*, *Δpil1* and *Δgup1Δpil1* strains. Cells were grown on YPD until O.D. $_{600}$=1 and 10-fold serial dilutions of each culture were spotted on YPD + 1 M NaCl or YPD + 1.5 M KCl. Results were scored after 3 days incubation at 30˚C. One representative experiment is shown.

Acetic acid-induced cell death process

Acetic acid triggers an apoptotic-like cell death in *S. cerevisiae* that resembles mammalian apoptosis (Ludovico *et al.*, 2001). G*UP1* deleted mutant was found to be

more sensitive to acetic acid-induced cell death, although dying with features of non-programmed/regulated cell death (Tulha *et al.*, 2012). This issue was discussed in detail in Chpt. 3. The *PIL1* deleted strains were subsequently tested in that regard.

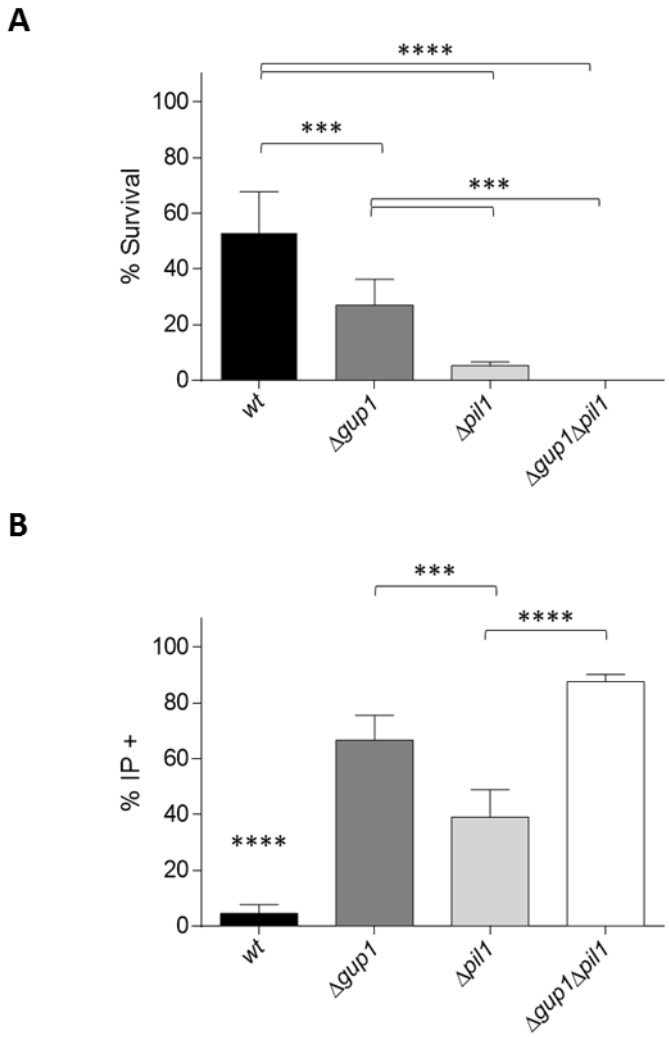

Figure 6 - *S. cerevisiae* BY4741 *wt, Δgup1, Δpil1 and Δgup1Δpil1* strains response to acetic acid-induced cell death. Exponentially growing cells on YNB were treated with 150 mM acetic acid for 3h. **(A)** Viability was determined by c.f.u. assay (results were normalized with 100% survival corresponding to the total c.f.u. at T_0). **(B)** Graphic representation of the percentage of cells displaying positive PI staining. Data represent mean ± SD of at least 3 independent experiments. **** P < 0.0001; *** P < 0.001 one-way ANOVA followed by Tukey's test

The deletion of *GUP1* increased the sensitivity of the yeast to acetic acid (Fig. 6A), as expected (Tulha *et al.*, 2012). The *PIL1* deleted mutant was even more affected (Fig. 6A). Survival after 3 h of exposure to acetic acid was significantly lower than the one of *wt* cells. Consistently, the double mutant Δ*gup1*Δ*pil1* identically treated yielded almost 100% of cell death (Fig. 6A). Subsequently, the number of propidium iodide positive

(PI+) cells, under the same experimental conditions, was quantified (Fig. 6B), to assess membrane integrity. This serves as preliminary assay to discriminate between accidental or programmed cell death types (discussed in Chpt. 3). 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 programmed cell death event. The same was true for the Δ*pil1* mutant cells. The decrease in cell survival observed in Δ*gup1*, on the other hand, was accompanied by loss of plasma membrane integrity, (Fig. 6B) suggesting a non-regulated cell death as previously described (Tulha *et al.*, 2012). The double mutant showed an identical behaviour to the Δ*gup1* single mutant, displaying a massive increase of PI+ cells as well (Fig. 6B). Altogether, the results indicate that the deletion of *PIL1* alone or in combination with *GUP1*'s increased considerably the cell sensitivity to acetic acid, though, from the results obtained with the double mutant, it was not able to reverse the necrotic type of death previously observed in the Δ*gup1* strains.

The deletion of *PIL1* provokes the disorganization of ergosterol distribution in the plasma membrane (Walther *et al.*, 2006; Grossmann *et al.*, 2007, 2008), identically to the deletion of *GUP1* (Ferreira and Lucas, 2008). Rafts integrity and ergosterol distribution was suggested to be important for cell survival to acetic acid induced apoptotic cell death (Mollinedo, 2012; Tulha *et al.*, 2012). These could underlie the extreme sensitivity to acetic acid of the Δ*pil1* mutant. Moreover, as mentioned above, eisosomes structure are strictly linked to the phosphoinositide homeostasis (Fröhlich *et al.*, 2014; Kabeche *et al.*, 2014). Fröhlich and co-workers observed a significant increase in PI(4,5)P2 phospholipid levels in cells lacking *PIL1* (Fröhlich *et al.*, 2014). The levels of phosphoinositide were previously suggested to regulate apoptotic cell death in mammalian cells (Mejillano *et al.*, 2001). Moreover, there is ground to foresee a link between the function of Gup1 and the phosphoinositide metabolism as previously mentioned. Besides the deletion of key enzymes from the phosphoinositide metabolism, such as Pik1, Stt4, Sac1 and Mss4, provoke phenotypes very similar to the ones observed when *GUP1* is disrupted (Lucas *et al.*, 2016), the levels of phospholipids are lowered in Δ*gup1* mutant. Moreover, Δ*gup1* also present an increase of the diacylglycerol levels, a direct product of PI(4,5)P2 cleavage (Oelkers *et al.*, 2000)

Other phenotypes

S. cerevisiae laboratory strains grown in favourable conditions form smooth regular-shaped/roundish colonies, which makes the study of colony differentiation very difficult. Nevertheless, Granek and Magwene, (2010) showed that, when cells are starved for carbon source, the growth pattern is altered, and some strains are able to form complex structured colonies. Thus, the methodology developed by Granek and Magwene, (2010), consisting in carbon-starving the cells in solid media for 12 days in YPD with 1% dextrose, was used to test the ability of the *GUP1* and *PIL1* deleted mutants to differentiate into complex multicellular structures. All the mutant strains, single and double, and *wt* presented the same colony differentiation, exhibiting identical moderately irregular shape (Fig. 7A), when compared to YPD-grown cells that exhibit regular dull-shaped colonies (not shown).

In *C. albicans* Gup1 strongly interferes with the capacity to form *hairy* colonies in inducing conditions (Ferreira *et al.*, 2010). Concomitantly, *Ca*Gup1 also interferes with the ability of cells to adhere, invade, and consequently form a biofilm. Previously, it was verified that the *S. cerevisiae* Δ*gup1* mutant produced a loose biofilm-like mat with a soft jelly-like texture (Faria-Oliveira *et al.*, 2015). This indicates that the deletion of *GUP1* alters the composition of the extracellular matrix, causing the consistency of the *S. cerevisiae* biofilms to change drastically (Faria-Oliveira *et al.*, 2015). No differences regarding the amount of biofilm production were reported though. That was quantified in the present work for the first time, testing the yeasts ability to form mats as discussed in Chpt. 3 (Reynolds and Fink, 2001). Following these authors methodology, the strains were grown in a low agar medium (0.3%) during 12 days, to promote the grown of a thin flat mat that covers a large surface, and the mat diameter was measured (Fig. 7B). All strains presented the same mat diameter. The effect of *GUP1* deletion on biofilms formation was studied in *C. albicans*, where it was shown that the correspondent null mutant produces a lesser amount of biofilm at a very delayed pace (Ferreira *et al.*, 2010). These results were not mirrored in the present work, being the mats unaffected by either *GUP1* or *PIL1* mutations. Still, the biofilms produced by *S. cerevisiae* and *C. albicans* are of a different nature, namely in what regards filamentation. The absence of filamentation of *Ca*Δ*gup1* was considered to be the cause for the delay observed in this mutant ability to develop biofilms (Ferreira *et al.*, 2010). The ability to switch between yeast-to-hyphae is mandatory in *C. albicans* biofilm formation process (Nett and Andes,

2006), but the formation of pseudo-hyphae is not required for *S. cerevisiae* to form biofilms (Reynolds and Fink, 2001). The present results just allow to conclude that Pil1 does not interfere with the phenotypes of Gup1 that regard morphology or colonization.

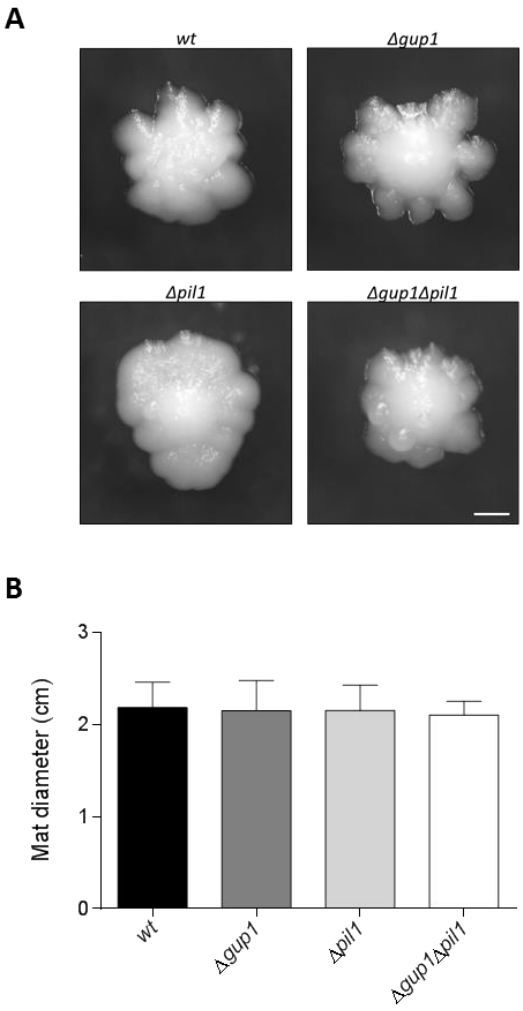

Figure 7 - Colony morphology and mat formation. (A) The colony morphology was visualized after a 10 days growth period in solid YPD (1% dextrose). One representative experiment is shown. **(B)** For the mat formation, overnight cultures were inoculated on YPD 0.3% agar plates, and results were scored, after 12 days of incubation at room temperature, measuring the diameter of mat. Results are representative of at least three independent experiments.

Conclusions

In this work, a new Gup1 physical partner was identified – the eisosome protein Pil1. Pil1 is a membrane associated protein that, together with Lsp1, forms the eisosome core component. Eisosomes delimit furrow-like plasma membrane invaginations associated

with MCC domains, that concentrate several specific proteins, as well as lipids (sterols) and signalling molecules. Although several functions were proposed for this structure, including protecting proteins from endocytosis, functioning as membrane reservoirs, maintaining phosphoinositide homeostasis, and regulating signalling responses, the discovery of the true function/s of eisosomes awaits further investigations.

It was observed that, in the absence of Gup1, the number of eisosome structures is reduced when compared to *wt*, though this is not a consequence of reduced *PIL1* expression, suggesting that Gup1 could be involved in eisosome formation. Pil1 preferentially binds to a minor phospholipid present in the membranes - PI(4,5)P2. Besides, the homeostasis of this lipid is also controlled by Pil1. The Δ*gup1* is affected in membrane composition, with a decrease in phospholipids and an increase in diacylglycerol (Oelkers *et al.*, 2000), which can be obtained by PI(4,5)P2 hydrolysis. Also, Δ*gup1* exhibits phenotypes very similar to those resulting from the deletion of enzymes involved in PI production. Thus, such alterations at the level of PI homeostasis could be the cause for the reduction in the number of eisosomes.

Moreover, the absence of Pil1 did not induce susceptibility to wall disturbing agents, or osmotic stress by high salt concentrations, which indicates that the wall was not affected by the deletion of *PIL1*. These results clearly reveal that the cell wall-related phenotypes are essentially related to the absence of Gup1, and justifies the fact that the double mutant was sensitive to CFW and CR while the single Δ*pil1* mutant was not. Accordingly, the aggregation/sedimentation was observed in Δ*gup1*Δ*pil1* but not in Δ*pil1* cells.

On the other hand, the Δ*pil1* mutant and, even more so, the double Δ*gup1*Δ*pil1* mutant were sensitive to SDS which primarily affects the membrane. This phenotype was not remediated by sorbitol, indicating that plasma membrane instability is the primary cause for such sensitivity. The deletion of both proteins induced an increased sensitivity to the membrane affecting detergent, which together with the previously described even distribution of ergosterol caused by the absence of each protein by itself (Walther *et al.*, 2006; Grossmann *et al.*, 2007, 2008; Ferreira and Lucas, 2008), supports the idea of important changes on Δ*gup1*Δ*pil1* plasma membrane, possibly regulated by the Gup1-Pil1 interaction.

The deletion of *PIL1* in a *GUP1* deleted genetic background, was not able to reverse the necrotic type of death previously observed in the Δ*gup1* strains, which indicates that

Gup1-Pil1 interaction is not relevant to determine the course of cell death. Still, the Δ*pil1* was also extremely sensitive to acetic acid-induced cell death. Such phenotype was never associated to the deletion of Pil1 before, neither to eisosomes functioning, and could be a consequence from the altered rafts distribution and/or increased phosphoinositol levels in Δ*pil1* mutant, as previously mentioned.

Importantly, the interaction of Gup1 with Pil1 could account for at least some of the biological functions of Gup1 while it locates in the plasma membrane, not necessarily overlapping with its functions in the ER or the mitochondria as discussed in Chpt. 3.

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CHAPTER 5

Phenotypic evaluation of Gup1 and Mep2 interaction

Abstract

The membrane-bound *O*-acyltransferase Gup1 was previously found to physically interact with the transceptor Mep2, causing the absence of Gup1 an increase of the Mep2 transport activity and associated signalling. Mep2 mediates the uptake of ammonium ion, but also functions as an ammonium sensor, regulating filamentous and invasive growth. The deletion of Gup1, also affects cell differentiation and invasiveness in *Candida albicans*. The physiological/biochemical role of Gup1 is though still unclear. The yeast Gup1 mammalian orthologue, HHATL, is a negative regulator of the post-translational lipidation of the Hedgehog morphogen and therein of the Hedgehog pathway. This lipidation in turn is operated by HHAT, the mammalian orthologue of yeast Gup2 close homologue Gup1. In yeasts, a paracrine pathway was never described. Ammonia secretion has been suggested to perform the distance cell-cell communication role instead. This secretion has though been described to be independent of Mep2. The role of Gup1 in yeast distance communication remain for the time being unknown.

The interaction between Mep2 and Gup1 was hereby studied. The double mutant Δ*gup1*Δ*mep2* was built and used in comparison with the correspondent single mutants to evaluate morphology/differentiation-related phenotypes, as well as the response to several stress-inducing agents. The localization of Mep2, assessed using a GFP chimera, differed in the Δ*gup1* background, accumulating asymmetrically in opposite direction from the budding site, while maintaining a granulated-type distribution observed in the *wt.* This altered Mep2 distribution might derive from or be associated with Δ*gup1* defects in the plasma membrane composition/organisation, cytoskeleton polarization and bud site selection. However, this would not explain straightforwardly the gain of function of Mep2 transporter and signalling observed when *GUP1* is deleted, demanding for a more direct involvement of Gup1 in Mep2 activity regulation. The Gup1 and Mep2 proteins, individually, appear to be essential to adherence/invasive growth, although the colony morphology and mat production did not mirror that need. Moreover, the deletion of Mep2 in Δ*gup1* increased the sensitivity to some cell wall related stress, namely CR and high temperatures, which might indicate that Mep2 associated transported and/or signalling could be important for cell survival when cell wall is affected.

Introduction

In 2011, Van Zeebroeck and colleagues screened a cDNA library for proteins interacting with Mep2 using a yeast two-hybrid system, and Gup1 was found (Van Zeebroeck *et al.*, 2011). Mep2 (methylammonium permease) protein belongs to the Amt/Mep/Rh family of transporters that are present in all kingdoms of life and include, *e.g.*, the prokaryotic AmtB proteins and the human Rhesus (Rh) blood group antigens (Andrade and Einsle, 2007). Fungi typically have more than one Mep paralogue. In *Saccharomyces cerevisiae*, three Mep proteins were described (Mep1, Mep2 and Mep3), and extensively characterized by kinetic, physiological, regulatory, biochemical and topological studies (Marini *et al.*, 1994, 1997; Marini and André, 2000; Rutherford *et al.*, 2008; Boeckstaens *et al.*, 2014; van den Berg *et al.*, 2016). The main physiological function of the Mep proteins is to mediate the uptake of ammonium ion (NH_4+) from the extracellular environment for biosynthetic purposes (Marini *et al.*, 1997). This is particularly important for cell survival when growing on poor ammonium conditions or other nitrogen sources (Boeckstaens *et al.*, 2007). From the three *S. cerevisiae* Mep proteins, the Mep2 is the one that displays the highest affinity for ammonium uptake (Marini *et al.*, 1997). Structurally, Mep2 was shown to associate in multimeric complexes (Rutherford *et al.*, 2008), which is in agreement with the 3D view of *Escherichia coli* AmtB and human RhCG forming trimers (Blakey *et al.*, 2002; Khademi *et al.*, 2004; Zheng *et al.*, 2004; Andrade *et al.*, 2005; Gruswitz *et al.*, 2010). In fact, yeast Mep2 proteins also form stable trimers, with each monomer having 11 transmembrane helices and a central pore for the transport of ammonium (van den Berg *et al.*, 2016). There is no indication available in the literature regarding a putative influence of the multimeric arrangement on each monomer transport activity. Yeast Mep2, however, has a considerable difference from all other ammonium transporters structure: under nitrogen-sufficient conditions, Mep2 is kept in a closed inactive conformation (van den Berg *et al.*, 2016). Moreover, Mep2 has an intracellular C-terminal tail and an extracytosolic N-terminus, which is unusual for a plasma membrane transporter (Marini and André, 2000). Besides, Mep2 is the only yeast Mep that is N-glycosylated in the outward-directed N-terminus, though this glycosylation is apparently not required for Mep2 functional activity (Marini and André, 2000). Ammonium transport is tightly regulated. Recently, Boeckstaens and co-workers

demonstrated that Mep2 is phosphorylated by the TORC1 effector kinase Npr1 under nitrogen-limiting conditions (Boeckstaens *et al.*, 2014). This phosphorylation on Ser457 within the C-terminal region of Mep2 was proposed to cause Mep2 "opening", i.e., the initiation of transport activity (Boeckstaens *et al.*, 2014; van den Berg *et al.*, 2016).

Besides ammonium transport activity, Mep2 has been suggested an additional function as ammonium receptor/sensor required for filamentous and invasive growth (Lorenz and Heitman, 1998a; Van Nuland *et al.*, 2006; Boeckstaens *et al.*, 2007; Rutherford *et al.*, 2008). This double function as transporter and sensor/receptor yielded the categorization of Mep2 as a transceptor, suggested for the first time by (Lorenz and Heitman, 1998a). As is the case of other transceptors (Kriel *et al.*, 2011), it is not clear how Mep2 interacts with downstream signalling partners, but the PKA, RAS-cAMP and MAPK pathways have been proposed as responding to Mep2 (Lorenz and Heitman, 1998a; Gagiano *et al.*, 1999; Van Nuland *et al.*, 2006; Rutherford *et al.*, 2008).

Gup1 is the yeast orthologue of HHATL, the mammalian negative regulator of Hedgehog (Hh) pathway (Abe *et al.*, 2008). HHATL inhibits the palmitoylation of the secreted Hh morphogen, which is operated by HHAT (Buglino and Resh, 2008), the mammalian counterpart of yeast Gup2. This pathway is extremely important in high eukaryotes for the regulation of cell growth and differentiation during embryogenesis or tissue healing. It operates through the secretion of a signalling protein that diffuses through the ECM and, upon recognition by a cell surface receptor, commands another cell behaviour (for a review see (Varjosalo and Taipale, 2008). The secreted signal is thus a morphogen and requires complex post-translation modifications, including lipids addition to the protein termini, to be secreted and diffuse regularly. One of those modifications is the palmitoylation of the N-terminal by the orthologue of Gup2 HHAT, inhibited by the orthologue of Gup1 HHATL (Abe *et al.*, 2008; Buglino and Resh, 2008). The pathway therefore depends on the diffusional properties of the secreted morphogen, which in turn depends on the modifications of the Hh signal. Such a pathway was never described in yeast. Yeast, like all microorganisms, is mostly know for living a planktonic life, but it also aggregates into organized multicellular structures, like colonies or biofilms, harbouring millions of cells that behave differentially for the common survival and reproduction. These aggregates organize through the secretion of an ECM that shares with the higher Eukaryotes a glycosidic and highly proteinaceous nature (Faria-Oliveira *et al.*, 2014, 2015a, 2015b). The key feature though, *i.e.*, a

hedgehog-like signal that mediates the communication between cells, has not been found yet.

Yeast cells in community, possess short-range intra-colony cell–cell as well as longer-range inter-colony communication. Long-distance signal between neighbouring colonies, according to Palková *et al.* (1997), is obtained through the active secretion a simple volatile alkaline compound, ammonia (NH_3). This long-distance signal is produced by colonies and transmitted in the form of pulses, and is capable of conditioning and synchronizing the growth of neighbouring colonies (Palková *et al.*, 1997). The first pulse produced by a colony is non-directed and promotes the acidification of the medium, while the second pulse is bigger and directed towards a neighbour colony (Palková *et al.*, 1997). This way, an ammonia long-range signal might warn a given colony for the presence of a nearby neighbouring colony, thus functioning as an alarm system for incoming competition for nutrients and, eventually, starvation. Upon this signal, changes in colonies that are important for their long-term survival are induced, promoting the reprogramming of cell metabolism and inducing colony morphological changes including inhibiting the growth of facing parts of two neighbouring colonies, directing the growth to free space (Palková *et al.*, 1997; Palková and Vachova, 2003). Interestingly, the ammonia diffusion inside a colony is also associated with its stratification into two different layers of metabolically distinct upper (U) and lower (L) cells (Cáp *et al.*, 2012). U cells are stress-resistant cells with a longevity phenotype, active TORC1 and autophagy, undergoing aerobic glycolysis, and releasing high levels of ammonia (Cáp *et al.*, 2012), resembling mammalian tumour cells (DeBerardinis and Cheng, 2010; Cáp *et al.*, 2012). On the other hand, L cells exhibit features of starving cells, sensitive to stress and losing viability more quickly during colony aging than U cells (Cáp *et al.*, 2012). L cells are involved in the release of nutrients to feed long-lived U cells (Cáp *et al.*, 2012).

How ammonium is produced by yeast and excreted out of yeast cells remains unclear. In addition to NH_3 diffusion, ammonium (NH_4^+) leakage might also occur through yet unidentified protein mediated pathways. The Ato proteins (Ato1, 2 and 3) were initially proposed to act as outward ammonium transporters (Palková *et al.*, 2002), however, they were latter described as acetate transporters (Paiva *et al.*, 2004), suggesting that their involvement in ammonium excretion might be indirect. On the other hand, electrophysiological studies revealed a non-selective cation channel (Nsc1)

displaying conductance for NH_4^+ (Bihler *et al.*, 1998). Finally, ammonium could also be loaded into vesicles via unknown transport pathways and then be released into the extracellular space by exocytosis, as occur for the excess of intracellular amino acids (Velasco *et al.*, 2004). Bulk excretion of ammonium followed by regulated retrieval by the Mep2 proteins has been described to happen in cells growing in several nitrogen sources (Boeckstaens *et al.*, 2007). It is assumed that the regulated retrieval by Mep2 could contribute to a more efficient control of the internal ammonium concentration, and thus of ammonium assimilation. It is fair to presume that it could also relate to the ammonia long-distance signalling. However, several observations indicate that ammonia pulse production is independent of external concentrations of ammonium, and that neither the transport by the ammonium permeases Mep1, Mep2, and Mep3, nor the sensing of the external ammonium by Mep2 seems to be important for ammonia signalling in yeast colonies (Palková *et al.*, 1997; Zikánová *et al.*, 2002). Instead, several observations support the hypothesis that the source of ammonia produced by colonies is the extracellular amino acids and their uptake (Zikánová *et al.*, 2002). The absence of another transceptor Gap1 (general amino acid permease), decreased ammonia production and excretion and consequent distance signalling, while mutation in the SPS amino acid sensor, completely abolished it (Zikánová *et al.*, 2002).

The present work characterized the interaction between Gup1 and Mep2 in *S. cerevisiae* by investigated some of the numerous Gup1-associated phenotypes. Mep2 distribution in the plasma membrane was found to be granulated, and in the *GUP1*-deleted background, asymmetric, concentrating oppositely to the budding site. Both proteins seem to be essential for adherence/invasive growth. In addition, the deletion of Mep2 in the Δ*gup1* background increased the sensitivity of the cell to wall stress-inducing agents and conditions exhibited by the Δ*gup1* mutant. This work also showed that some of the phenotypes previously described in association to *GUP1* were undisturbed by low-ammonium medium, being therefore not dependent on nitrogen availability.

Material and Methods

Strains and growth conditions

The bacterium and yeast strains used in this study are listed in Table 1.

Table 1 - Microbial strains used in the present study.

Strain	Genotype	Source
S. cerevisiae BY4741 *wt*	*MATa; ura3Δ0; leu2Δ0; his3Δ1; met15Δ0*	Euroscarf
S. cerevisiae BY4741 Δ*gup1*	*MATa; ura3Δ0; leu2Δ0; his3Δ1; met15Δ0; YGL084c::kanMX4*	Euroscarf
S. cerevisiae BY4741 Δ*mep2*	*MATa; ura3Δ0; leu2Δ0; his3Δ1; met15Δ0; YNL142W::kanMX4*	Euroscarf
S. cerevisiae BY4741 Δ*gup1*Δ*mep2*	*MATa; ura3Δ0; leu2Δ0; met15Δ0; YNL142W::kanMX4; YGL084c::HIS3*	This study
S. cerevisiae BY4741 Δ*mep2 – MEP2-GFP*	*MATa; ura3Δ0; leu2Δ0; met15Δ0; YNL142W::MEP2-GFP-HIS3*	Huh *et al.*, 2003
S. cerevisiae BY4741 Δ*gup1*Δ*mep2 – MEP2-GFP*	*MATa; ura3Δ0; leu2Δ0; his3Δ1; met15Δ0; YNL142W::MEP2-GFP-HIS3; YGL084c::kanMX4*	This study
E. coli XL1 Blue	*endA1gyrA96 (nalR) thi-1 recA1 lac glnV44 F'[::Tn10 proAB+ lacIq Δ(lacZ)M15]hsdR17(rK- mK+)*	Stratagene

E. coli XL1 Blue was purchased from Stratagene. It was cultivated in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, (2% agar for solid medium) pH 7.2), liquid or solid, appropriately supplemented for antibiotic resistance with 100 mg/mL ampicillin when necessary. Cultivation of *E. coli* cells, as well as isolation and manipulation of plasmid DNA, were done using standard procedures (Ausubel *et al.*, 1999). Yeasts were cultivated on YPD (1% yeast extract, 2% peptone, 2% glucose, 2 (2% agar for solid medium)), or YNB medium (0.175% YNB without amino acids and nitrogen source (Difco), 0.5% $(NH_4)_2SO_4$, 2% glucose) appropriately supplemented according to auxotrophic requirements. For experiments with low nitrogen conditions, cells were cultured at 30ºC into exponential growth phase in YPD medium, harvested by centrifugation (5min at 5000 rpm), washed with water, and suspended in nitrogen starvation medium, SLAD (0.175 % YNB without amino acids and nitrogen source

(Difco), 50µM $(NH_4)_2SO_4$, 4 % glucose, appropriately supplemented according to auxotrophic requirements). Liquid cultures were performed in batch at 30ºC and 200 rpm orbital shaking in a 1/3 air to liquid ratio.

Construction of *S. cerevisiae* Δ*gup1*Δ*mep2* double mutant and Δ*gup1*Δ*mep2-MEP2-GFP*

The double mutant of *S. cerevisiae* Δ*gup1*Δ*mep2* was constructed replacing the *GUP1* gene in BY4741 Δ*mep2* (Euroscarf) with a *gup1::HIS3* disruption cassette, amplified by PCR from the p416 plasmid (Addgene) with the primers A and B listed in Table 2. The *gup1::HIS3* disruption cassette was used to transform BY4741 Δ*mep2* strain by homologous recombination using standard protocols (Ito *et al.*, 1983). The generated transformants were selected in YNB medium without histidine. Positive clones were confirmed by colony PCR using the *GUP1* deletion confirmation primers E and F listed in Table 2.

Table 2 - Primers used in the present study and their sequence.

Name	Primer
A - Fw *gup1::HIS3* cassette	5'ATGTCGCTGATCAGCATCCTGTCTCCCCTAATTACTTCCGTTTC CCGCAATTTTCTTTTTC 3'
B - Rv *gup1::HIS3* cassette	5'TCAGCATTTTAGGTAAATTCCGTGCCTCTTTTCTTCTTCTATAT ATATCGTATGCTGCAGC 3'
C - Fw *gup1::KanMx* cassette	5'ATGTCGCTGATCAGCATCCTGTCTCCCCTAATTACTTCCGGAC ATGGAGGCCCAGAATAC 3'
D - Rv *gup1::KanMx* cassette	5'TCAGCATTTTAGGTAAATTCCGTGCCTCTTTTCTTCTTCTCAGT ATAGCGACCAGCATTC 3'
E - Fw *GUP1* deletion confirmation	5' ATCAGCTCAATCGGACATA 3'
F - Rv *GUP1* deletion confirmation	5' ATCATATGGTCCAGAAACC 3'
G - Rv *GUP1* deletion confirmation	5' CTGCAGCGAGGAGCCGTAAT 3'

The construction of the BY4741 Δ*gup1*Δ*mep2-MEP2-GFP* was performed deleting the *GUP1* gene in the BY4741 Δ*mep2-MEP2-GFP* strain (kindly provided by Erin K. O'Shea, Howard Hughes Medical Institute (Huh *et al.*, 2003)). The *GUP1* gene was deleted using the KanMx disruption cassette, amplified from pUG6 plasmid (Addgene) with the primers C and D listed in Table 2. The *gup1::KanMx* disruption cassette was

used to transform BY4741 Δ*mep2-MEP2-GFP* strain by homologous recombination using standard protocols (Ito *et al.*, 1983). Transformants were selected in YNB medium with geneticin (200 mg/L). Positive clones were confirmed by colony PCR using *GUP1* deletion confirmation primers E and F listed in the Table 2.

Total RNA isolation

Nitrogen-starved cells were collected (~$5x10^7$ cells), spun down, and the pellets were frozen in liquid nitrogen and stored at -80ºC. Samples were mechanically disrupted using glass beads in a swing-mill at 30 Hz for 15 min. Total RNA was extracted and isolated using the NucleoSpin® RNA kit (Macherey-Nagel), and subsequently quantified in a ND-1000 UV-visible light spectrophotometer (NanoDrop Technologies). RNA quality was evaluated by agarose-gel electrophoresis. A negative control consisted in verifying the absence of contaminant gDNA by directly using the isolated RNA as template for the amplification reaction.

Quantitative Real Time-PCR (qRT-PCR)

Primers for qRT-PCR (Table 3) were built using Primer3Plus software, aligned against *S. cerevisiae* genome sequence (http://www.yeastgenome.org/blast-sgd) for specificity confidence, and analysed with the Mfold server (http://unafold.rna.albany.edu/?q=mfold) to check for the possible formation of self-folding secondary structures. 500 µg of total RNA was reverse-transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad). The cDNA levels were then analysed using the Bio-Rad® CFX96 Touch™ real-time PCR instrument. Each sample was tested in duplicate in a 96-well plate (Bio-Rad, CA). The reaction mix (10 µL final vol.) consisted of 5 µL of SsoAdvanced™ SYBR® Green Supermix (Bio-Rad), 0.25 µL of each primer (250 nM final concentration) and 1 µL of cDNA preparation. A blank (no template) control was included in each assay. The thermocycling program consisted of one hold at 98°C for 30 sec, followed by 40 cycles of 10 sec at 98°C and 20 sec at 60°C. After completion of these cycles, a melting-curve was performed (65°C-95°C, 0.5°C increments, 3s) and data collected to verify PCR specificity and the absence of primer dimers. The data were normalized to *18S* gene. The comparative Ct method analysis ($2^{-\Delta\Delta CT}$ method) (Schmittgen and Livak, 2008) was used to analyse the results. The

results presented are the mean of the three different extractions of total RNA, analysed by at least duplicate PCRs.

Table 3 - qRT-PCR primers used in the present study and their sequence.

Name	**Primer**
Fw *GUP1* qRT-PCR	5' GCGTGGGAAAATGACACAC 3'
Rv *GUP1* qRT-PCR	5' AAACAGCCTCCACGGAATC 3'
Fw *MEP2* qRT-PCR	5' CAGATGCGGAAGAAAGTGG 3'
Rv *MEP2* qRT-PCR	5' ACAACGGCTGACCAGATTG 3'
Fw *18S* qRT-PCR	5' TGCGATAACGAACGAGACC 3'
Rv *18S* qRT-PCR	5' TCAAACTTCCATCGGCTTG 3'

Microscopy procedures

Fluorescence microscopy. The sub-cellular localization of Mep2-GFP chimera was assessed in exponential cells grown in YNB low-nitrogen medium. Cells were observed in a Leica Microsystems DM-5000B epifluorescence microscope with the appropriate filter settings, using a 100X oil-immersion objective. Images were acquired through a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software. At least 200 cells were observed in each experiment.

Light microscopy. (i) Cells were observed by light microscopy (LM) in mid-exponential yeast cultures grown in YNB low-nitrogen medium. Microscopy assessments were done in a Leica Microsystems DM-5000B epifluorescence microscope. Images were acquired through a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software. (ii) To observe colony morphology/differentiation, mid-exponential yeast cultures were diluted 100x, spotted (50 µL) onto YPD (1% glucose), and incubated for 12 days at room temperature. Resulting colonies were visualized in a Leica Zoom 2000 stereo-microscope and the images were acquired through a Leica EC3 digital camera and processed with LAS AF Leica Microsystems software.

Mat formation

Mat formation was assessed as previously described (Reynolds and Fink, 2001) with some modifications. Overnight cultures were collected by centrifugation, diluted to a final $O.D._{600}$=1 in water and 5µl of this suspension was used to inoculate 0.3% agar YPD plates (all plates were prepared at the same time (one day before) to ensure the same level of medium hydration, and therefore the same medium solidity). The plates were then sealed with parafilm and incubated at room temperature. Results were scored after 12 days of incubation by measuring the diameter of the colony formed.

Phenotypic assessment

Culture sedimentation. To assess the propensity of a yeast culture in suspension to sediment (Ferreira *et al.*, 2006), cells were grown to mid-exponential phase in SLAD medium at 30ºC, collected by centrifuging 5 min at 5000 rpm and resuspended to $O.D._{600nm}$=1, and left for 20 min at room temperature without shaking to allow sedimentation. After this period, the resultant cell suspension was photographed.

Drop tests. Drop tests were performed using cell suspensions at an $O.D._{600nm}$=1, collected from mid-exponential grown cultures. A serial dilution of 4x100 was made, and 5 µL of each suspension was applied on solid medium. Results were scored after 3 days of incubation at 30ºC, unless otherwise stated.

Adherence/Invasive growth. To assay the ability of medium adherence/invasion, cells were collected from mid-exponential grown cultures to a final $O.D._{600nm}$=1 spotted on SLAD plates and incubated at 30 °C for 6 days (Rutherford *et al.*, 2008). The grown colonies were challenged with washing under tap water to evaluate their adherence ability.

Results and Discussion

MEP2 expression and protein localization

The expression of all three *MEP* genes, including *MEP2*, is submitted to nitrogen control and is highest during growth under poor nitrogen supply (Marini *et al.*, 1997). Therefore, all the assays described in this work were performed in a low-ammonium medium (SLAD), unless otherwise mentioned. The *MEP2* expression levels, as well as the plasma membrane localization of Mep2 in nitrogen-starved cells, were assessed by RT-PCR or by fluorescence microscopy, respectively (Fig. 1). The *MEP2* expression in the Δ*gup1* mutant was similar to the expression observed in *wt* cells (Fig. 1A).

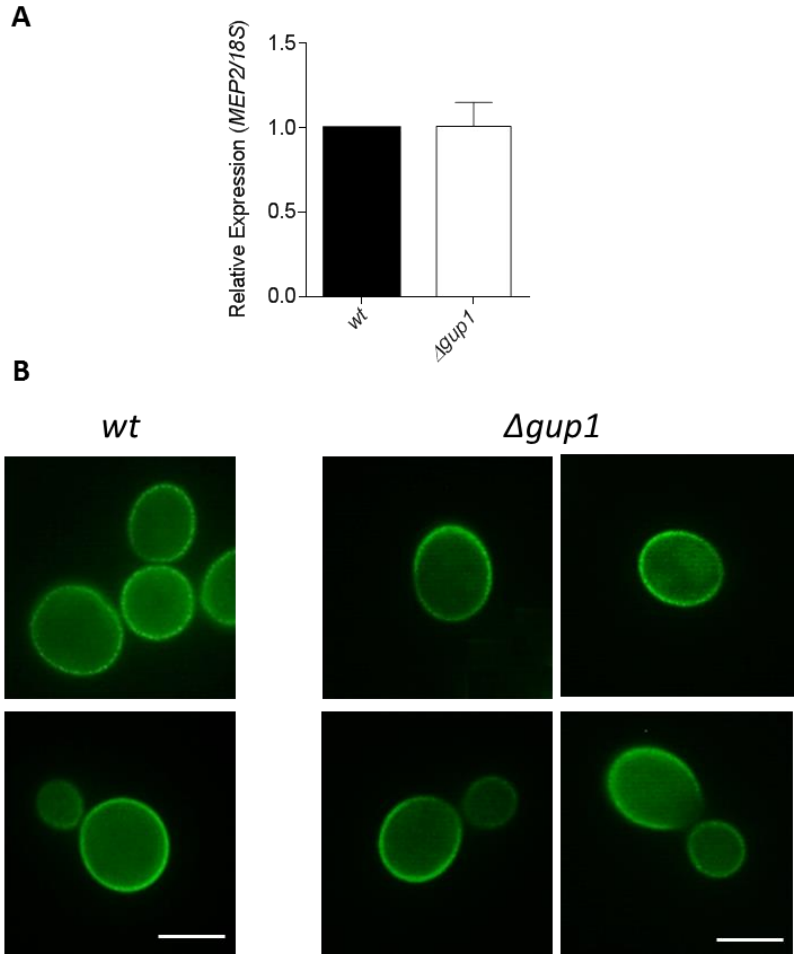

Figure 1 – *MEP2* expression (A) and localization (B) in *S. cerevisiae* BY4741 *wt* and *Δgup1* strains cultivated in nitrogen starvation media (SLAD). (A) Relative expression of *MEP2* by qRT-PCR. *MEP2* expression was normalized against *18S* and represented relative to the levels in the *wt* strain as calculated by the comparative Ct method analysis ($2^{-\Delta\Delta CT}$ method). **(B)** The Mep2 localization was assessed using the *wt* and Δ*gup1* strains with a chromosomal Mep2-GFP insertion. Results are representative of at least three independent experiments.

Mep2 distribution has been described by Van Zeebroeck *et al.* (2011) as continuous throughout the whole plasma membrane, while the same authors claimed that the Mep2 distribution became patched in Δ*gup1*. In the present work, results showed otherwise, that Mep2 distribution through the whole plasma membrane of *wt* cells is instead granulated (Fig 1B), which is more compatible with the described multimeric complex association of these transceptors (van den Berg *et al.*, 2016). Identically, also a plasma membrane punctated distribution of Mep2 was described by Spira *et al.* (2012). On the other hand, the *GUP1* deleted mutant now assessed presented the same granulated pattern, though not throughout the entire plasma membrane, while it appears that the fluorescence concentrates away from the budding site (Fig 1B). Therefore, Gup1 seems to be required for regular continuous distribution of Mep2, along the plasma membrane. Various phenotypes of the Δ*gup1* strain could contribute to this irregular localization pattern, including the modified plasma membrane composition (Oelkers *et al.*, 2000), the defective secretory pathway (Bonangelino *et al.*, 2002), and the abnormal cytoskeleton polarization and budding site selection (Ni and Snyder, 2001; Casamayor and Snyder, 2002). Moreover, in the absence of Gup1, lipid rafts become less stable and less numerous (Ferreira and Lucas, 2008), which inevitably affects the membrane distribution of the associated proteins. Van Zeebroeck *et al.* (2011) considered this as a possible justification for their observed alterations of Mep2 distribution in the *GUP1* mutant. Still, Mep2 has not been described as a raft protein, and do not co-localize with the lipid raft resident protein Pma1 (Spira *et al.*, 2012), therefore the influence of rafts disruption in Mep2 distribution is unlikely.

Van Zeebroeck *et al.* (2011) described that Mep2-mediated transport and signalling was considerably increased in Δ*gup1* mutant. An uneven distribution of Mep2 on the plasma membrane of Δ*gup1* cells cannot straightforwardly explain this result. The authors hypothesised that that the higher levels of Mep2 transport and signalling activity could result from an enhanced level of the transceptor protein at the plasma membrane, however, no experimental evidences supporting this hypothesis was presented (Van Zeebroeck *et al.*, 2011).

There is one interesting example in the literature, of transporters that aggregate to form functional structures that promote a very rapid flux of substrate, the Ca^{2+} receptor/channels from myocytes (Jayasinghe *et al.*, 2009; Hou *et al.*, 2015). These participate in the contraction of cardiac muscle which depends on the fine control of fast

massive in- and outward Ca^{2+} fluxes. The spatial location and clustering of calcium channels, like for example ryanodine receptors (RyRs) (Hou *et al.*, 2015) is long thought to be crucial for the compartmentalization of Ca^{2+}. The large structure of clustered RyRs allow, extremely fast Ca^{2+} release sparks, which create waves of Ca^{2+} spreading throughout the tissue (Langer and Peskoff, 1996; Soeller and Cannell, 1997; Goldhaber *et al.*, 1999). RyRs clusters are visible as punctate immunofluorescence images at the plasma membrane of several organisms' cardiac myocytes (Soeller *et al.*, 2007; Hayashi *et al.*, 2009). The Mep2 uneven distribution presently observed in the absence of Gup1 could identically promote a faster transport, with possibly different kinetic characteristics. A possible role for Gup1 as a negative Mep2 regulator, direct or indirect, cannot though be presently excluded.

Morphology and differentiation phenotypes

Both Gup1 and Mep2 proteins were previously associated to several morphological and differentiation processes, including filamentation, adherence/invasive growth, colony morphology and mat/biofilm formation (Lorenz and Heitman, 1998a, 1998b; Gagiano *et al.*, 1999; Rutherford *et al.*, 2008; Ferreira *et al.*, 2010; Faria-Oliveira *et al.*, 2015a, 2015b). Therefore, the implications of the Gup1 and Mep2 interaction (Van Zeebroeck *et al.*, 2011) on these morphological and differentiation phenotypes was assessed. For that purpose, a double mutant Δ*gup1*Δ*mep2* was generated. As mentioned above, all the phenotypes were tested using cells cultivated in SLAD medium, *i.e.*, under poor ammonium/nitrogen conditions. This medium was chosen bearing in mind that Mep2 is an ammonium permease that is only expressed under low ammonium concentrations (Marini *et al.*, 1997). TORC1 complex signalling is the central coordinator of physiological responses of the cell to changes in nitrogen source and availability (De Virgilio and Loewith, 2006). The involvement of Gup1 in this pathway was previously suggested (Lucas *et al.*, 2016), yet, SLAD-cultivated cells were never previously used to test *GUP1*–related mutants, so the influence of the nitrogen availability on Gup1 function was hereby also assessed and discussed.

As already mentioned and discussed in Chpt. 3 and 4, yeast laboratory strains usually do not exhibit complex colony morphology. This is well-known in yeast-laboratory practice, and does not depend on the use of rich or poor media such as YPD or YNB (Palková, 2004). Instead, they do it profusely when starved for carbon (Granek and

Magwene, 2010). In these conditions, colonies of well-known and widely used laboratory strains develop complex, organized, macroscopic structures. The derived *morphotypes* fall into several categories: spokes (with concentric rings), lacy, coralline, mountainous or irregular (Granek and Magwene, 2010). The *S. cerevisiae wt* and Δ*gup1* strains, from the genetic background used in the present work, as well as the previously used W303 background, did not display complex colonies in any of the culture media, temperature or stress conditions tested (*e.g.* Ferreira *et al.*, 2006 or Ferreira and Lucas, 2008). Unlike *S. cerevisiae*, *C. albicans GUP1* null mutant displayed a severe colony morphology phenotype, corresponding to losing the ability to form *hairy* colonies with aerial hyphae in spider medium (Ferreira *et al.*, 2010). This was associated to the loss of yeast-hyphae transition, which is central to the invasiveness and virulence behaviour of this yeast and is severely affected by the deletion of C*aGUP1* (Ferreira *et al.*, 2010). Concomitantly, the mutant displayed a weak and highly delayed ability to develop biofilm (Ferreira *et al.*, 2010). Interestingly, mouse *GUP1*/HHATL (Abe *et al.*, 2008) cDNA was able to complement hyphae development defects of *ca*Δ*gup1*null mutant (Lucas *et al.*, 2016). On the other hand, the *S. cerevisiae GUP1* cDNA only partially complements the absence of this gene, since caΔ*gup1*mutant expressing *ScGUP1* is able to start developing filamentous cells, but unable to complete that differentiation (Armada, 2011).

The colony morphology of the *wt*, Δ*gup1*, Δ*mep2* and Δ*gup1*Δ*mep2* strains was firstly observed in cells cultivated in SLAD media. In this media, the *wt* and Δ*gup1* strains, as well as the *MEP2* deleted strains, displayed regular dull-shaped colonies (not shown). Subsequently, the same strains were starved for carbon in solid media (12 days in YPD 1% dextrose) (Granek and Magwene, 2010) in order to promote the development of structured complex colonies as previously mentioned. The *wt*, Δ*gup1*, Δ*mep2* and Δ*gup1*Δ*mep2* strains all developed the same moderately irregular-shaped colonies (Fig. 2), which tended to get more irregular along time. No differences were therefore observed between the *wt* and the mutants (Fig. 2). The BY4741 genetic background, used in this study, is different from the strains in which this methodology was demonstrated (Granek and Magwene, 2010). As discussed in Chpt. 3, *S. cerevisiae* BY4741 *wt* has a mutation in the *FLO8* transcriptional activator that results in a lower expression of GPI-anchored flocculin *FLO11* (Liu *et al.*, 1996). Flo11 is important for the cell–cell and cell-surface adhesion that are required for pseudo-hyphal

differentiation, biofilm formation, flocculation, and also for the formation of structured colonies (Lambrechts *et al.*, 1996; Reynolds and Fink, 2001; Ishigami *et al.*, 2004; Verstrepen *et al.*, 2004; Granek and Magwene, 2010; St'ovíček *et al.*, 2010; Vopálenská *et al.*, 2010). This could explain why the *wt* BY4741 was unable to develop such type of colonies (Granek and Magwene, 2010).

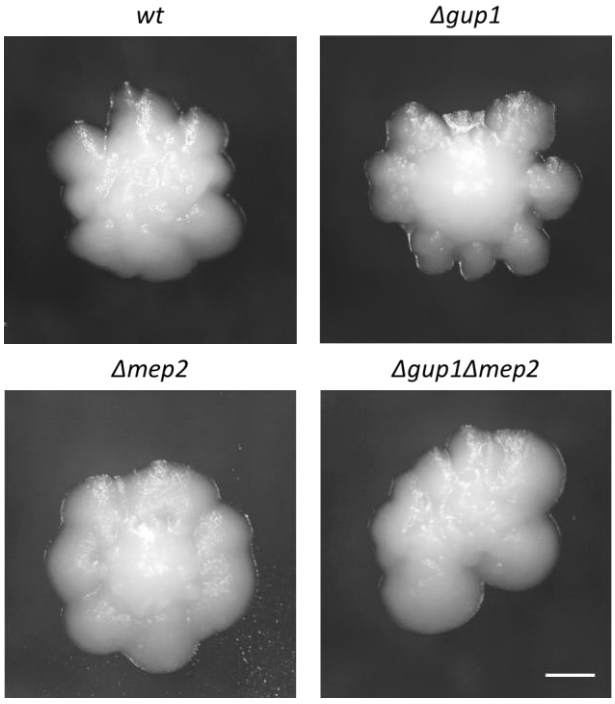

Figure 2 - Colony morphology of *S. cerevisiae* BY4741 *wt, Δgup1, Δmep2 and Δgup1Δmep2* strains. The colony morphology was visualized after a 10 days growth period in solid YPD (1% dextrose). One representative experiment is shown.

The factors underlying the differences in the morphology of a yeast colony are not well understood, in spite of all the efforts so far (Palková and Forstová, 2000; Kuthan *et al.*, 2003; Váchová and Palková, 2005; Granek and Magwene, 2010; Cáp *et al.*, 2012). Empirically, it is considered that a complex macroscopic colony should imply differentiation at cellular level, switching from yeast cells to hyphae or pseudo-hyphae. Besides, complex colonies generally produce an extensive extracellular matrix (ECM) that is absent from simple colonies (Kuthan *et al.*, 2003). *S. cerevisiae* cannot form true hyphae but it can differentiate into pseudo-hyphae, which is not easily assessed in haploid yeast cells. Still, growth on low-ammonium medium triggers

adherence/invasion, a behaviour similar to the pseudo-hyphae growth of diploid *S. cerevisiae* (Gimeno *et al.*, 1992; Roberts and Fink, 1994; Cullen and Sprague, 2000). Unlike *C. albicans*, this was never tested in *S. cerevisiae* in association with the *GUP1* deletion. On the other hand, Mep2 was showed to be required, under nitrogen limiting conditions, for pseudo-hyphae differentiation, adherence and invasive growth, both in *C. albicans* (Biswas and Morschhäuser, 2005) and *S. cerevisiae* (Lorenz and Heitman, 1998a, 1998b; Gagiano *et al.*, 1999; Rutherford *et al.*, 2008). Moreover, yeast differentiation, besides promoting adherence/invasion, also promotes biofilm formation. This was assessed indirectly by comparing the ability of the *wt*, Δ*gup1*, Δ*mep2* and Δ*gup1*Δ*mep2* strains (i) to adhere correctly to the surface of the agar, and (ii) to form thin mats (Reynolds and Fink, 2001), as a means to quantify the ability of a strain to colonize the environment. *i.e.*, mimicking the ability to produce a biofilm as discussed in Chpt. 3.

The ability of the cells to adhere to agar surfaces was tested as previously in SLAD medium (Gimeno *et al.*, 1992; Roberts and Fink, 1994; Rutherford *et al.*, 2008). Growth of *wt* cells in this medium resulted in adherence to the agar surface (Fig. 3), which was absent from both Δ*gup1* and Δ*mep2* mutants, as well as the double mutant Δ*gup1*Δ*mep2* (Fig. 3). This shows that *GUP1* deletion doesn't complement the loss of adherence described for Δ*mep2* mutant, suggesting that both proteins could have equivalent roles in the invasive/adherence process.

To test the ability to develop thin mats, yeasts were cultivated for 12 days on low agar (0.3%) YPD, as in Chpt. 3 and 4, or alternatively on low agar (0.3%) SLAD medium, after which the mat diameter was measured (Reynolds and Fink, 2001). As demonstrated in the Fig. 4, the ability of each strain to form a flat mat was identical in YPD, as shown by the equal mat diameter. Growth on SLAD medium did not allow the development of mats of any strain, presenting a much smaller mat diameter when compared to that observed in YPD medium (not shown). Therefore, though the results in YPD suggest that the interaction between Gup1 and Mep2 is not important for mat production, the doubt remains, since the expression levels of Mep2 in that medium are expected to be too low.

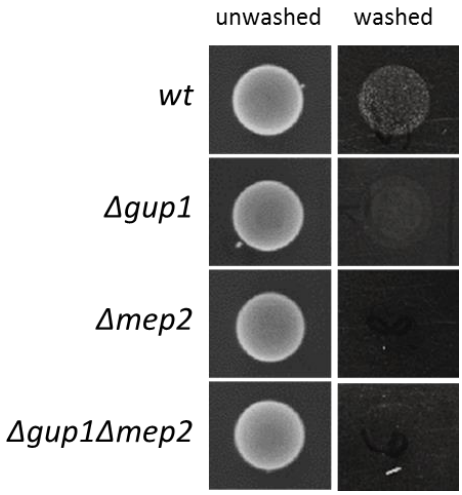

Figure 3 – Adherence/invasive growth. Yeast strains were grown on YPD until mid-exponential phase and 10 µl of each culture were spotted onto SLAD medium. Results were scored after 6 days of incubation at 30ºC by washing the cells with constant flux of tap water. Left and right columns represent the unwashed and washed conditions, respectively. One representative experiment is shown.

In *S. cerevisiae*, several signal transduction pathways govern the yeast culture switch, in response to nutrient limitation, from budding yeast growth to pseudo-hyphae formation, adherence, and invasive growth. These include the MAPK, RAS/PKA, SNF, and TOR pathways (for a review see Cullen and Sprague, 2012). As mentioned before, Mep2 is required for filamentous and invasive growth, functioning as an ammonium sensor that governs cellular differentiation (Lorenz and Heitman, 1998b; Gagiano *et al.*, 1999; Biswas and Morschhäuser, 2005; Rutherford *et al.*, 2008). Nevertheless, the ammonium transport activity through Mep2 was demonstrated to be required, but not sufficient, to sense ammonia and induce filamentous and invasive growth (Boeckstaens *et al.*, 2007; Rutherford *et al.*, 2008). The molecular mechanisms that mediate Mep2 downstream signalling are yet not well understood. Several suggestions have been made: (i) the MAPK pathway through the MAP kinase-regulated transcription factor Ste12 (Lorenz and Heitman, 1998a; Rutherford *et al.*, 2008), (ii) the TOR pathway through TORC1, since it is known to regulate Mep2 transport activity through phosphorylation by the Npr1 kinase (Boeckstaens *et al.*, 2014), and (iii) RAS-cAMP (Lorenz and Heitman, 1998a). Notably, the Msn1 and Mss11 transcriptional regulators involved in, namely, filamentous growth and pseudo-hyphae differentiation, were shown to act downstream of Mep2 and Ras2 (Gagiano *et al.*, 1999).

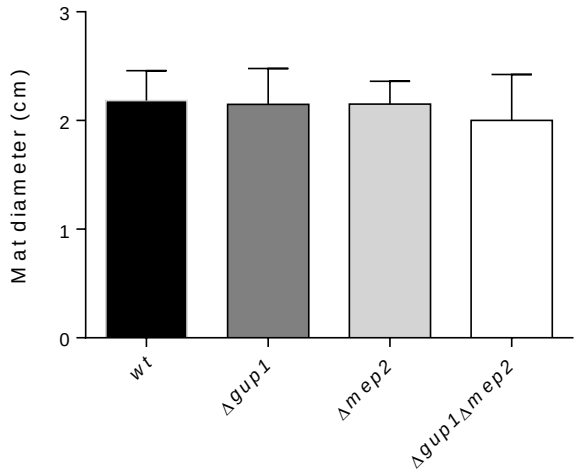

Figure 4 - Mat formation of *S. cerevisiae* BY4741 *wt, Δgup1, Δmep2 and Δgup1Δmep2* strains. Overnight cultures were inoculated onto YPD agar plates (0,3% agar), and results were scored after 12 days of incubation by measuring the diameter of mat. Results are representative of at least three independent experiments.

The absence of *GUP1* was previously showed to cause an increase of ammonium transport through Mep2, as well as signalling through protein kinase A (PKA) pathway by increasing trehalase activity (Van Zeebroeck *et al.*, 2011). This suggests that Gup1 protein might act as a Mep2 negative regulator. In such case, in the absence of Gup1, Mep2 should be uninhibited, and consequently its role in promoting adherence/invasion enhanced, which was not observed (Fig. 3). However, as mentioned above, the transporting function is necessary but not enough to induce Mep2 sensing and derived signalling (Rutherford *et al.*, 2008). Moreover, the Mep2 signalling through the cAMP-independent activation of the PKA pathway seems to be independent of its role in pseudo-hyphae and invasive growth (Van Nuland *et al.*, 2006). Therefore, the increased Mep2 transport and signalling through PKA pathway in the absence of Gup1, was not enough, or was not directed to promote adherence/invasive growth in Δ*gup1* mutant. Though a possible role of Gup1 in Mep2 downstream signalling cannot be discarded, the loss of adherence observed in Δ*gup1* mutant, on the other hand, could derive from its altered cell wall composition/architecture (Ferreira *et al.*, 2006). This was therefore subsequently assessed in the *MEP2* deleted backgrounds.

Cell wall and membrane associated phenotypes

S. cerevisiae Δ*gup1* mutant presents severely altered plasma membrane and cell wall composition and structure, resulting in a different response to several cell wall and membrane stress-inducing agents and culture conditions (Ferreira *et al.*, 2006). The simplest-associated phenotypes were assessed, leaving undone the wall and membrane chemical composition analysis.

Yeast aggregation. Yeast flocculation is the aggregation of single yeast cells, that usually causes a rapid sedimentation from the liquid culture (Zhao and Bai, 2009; Vidgren and Londesborough, 2011). The most common mechanisms of yeast flocculation are the lectin-mediated adhesion of adjacent yeast cells to form large cell aggregates (Miki *et al.*, 1982; Stratford, 1992). This aggregation occurs through the binding of flocculins present in at the surface of one cell to mannosides from the cell wall of adjacent cells. It was demonstrated that this binding typically occurs in a calcium-dependent manner (Miki *et al.*, 1982; Stratford, 1992; Veelders *et al.*, 2010). Therefore, the formation of these cell clusters is not an irreversible process and can be dissociated by the addition of a chelating agent, like EDTA that removes calcium ions, or by the addition of mannose, which competitively displaces cell wall mannose residues from flocculin binding sites. Yeast cells lacking Gup1 when grown in liquid media have a natural tendency to form aggregates that swiftly sediment (Ferreira *et al.*, 2006). This sedimentation was sensitive to EDTA and re-established by the subsequent addition of $CaCl_2$ (Ferreira *et al.*, 2006; Ferreira, 2005), though it was insensitive to the addition of mannose (Ferreira, 2005). Moreover, the levels of Flo1, the most abundant flocculin in *S. cerevisiae*, are unchanged in Δ*gup1* cells, when compared to *wt* (Ferreira *et al.*, 2006). This led the authors to propose that the sedimentation phenotype of Δ*gup1* does not seem to account for the described flocculation mechanisms (Ferreira *et al.*, 2006), thus some relation with the mutant changed cell wall molecular composition might exist. The possible interference of Mep2 protein in the aggregation phenotype associated to *GUP1* deletion was assessed. As expected, unlike *wt*, the Δ*gup1* strain displays cell aggregation and sedimentation in YPD, the same happening in SLAD media (Fig. 5). The Δ*mep2* and Δ*gup1*Δ*mep2* behaved identically to *wt* and Δ*gup1* strains, respectively (Fig. 5), indicating that the tendency to form large aggregates that swiftly deposit in the absence of Gup1 does not derive from a process dependent of the presence of Mep2 or its association with Gup1. Importantly, it does not depend on the

nitrogen availability either. This could mean that Mep2, unlike Gup1, does not affect the cell wall.

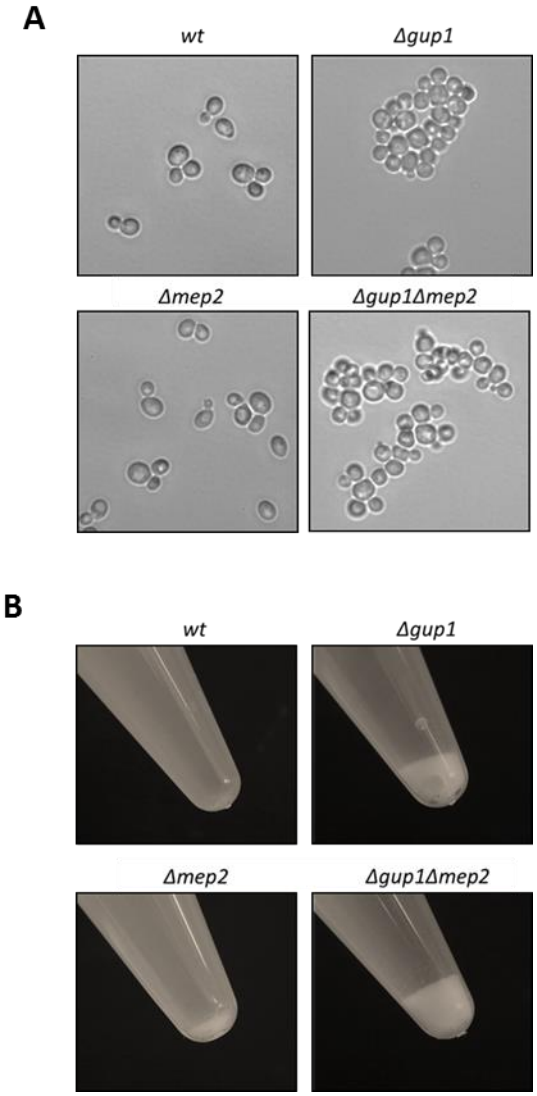

Figure 5 – Cell aggregation and sedimentation phenotypes. The assays were performed in mid-exponential cells grown in SLAD medium. **(A)** Cell aggregation ability was visualized directly by LM. **(B)** Sedimentation phenotype was recorded after leaving the culture to rest at room temperature for 20 min. One representative experiment is shown. Bar = 5µm

Growth at high temperature. All strains were exposed to growth at high temperatures (37ºC), a stress known to provoke mainly a cell wall stress (Kamada *et al.*, 1995; Mensonides *et al.*, 2005). On SLAD medium, Δ*gup1* and Δ*gup1*Δ*mep2* exhibited decreased growth ability at 37ºC (Fig. 6 – right panel) in comparison to the control at 30°C (Fig. 6 – left panel), more evident in the double mutant. On the other hand, Δ*mep2* behaved identically to *wt* strain. Previous results had shown that Δ*gup1* mutant

cultivated on rich YPD medium (Ferreira *et al.*, 2006) was sensitive to 37°C. A high temperature sensitive phenotype is generally considered an indirect consequence of altered cell wall composition (de Nobel *et al.*, 2000), as proved to be the case of the Δ*gup1* mutant (Ferreira *et al.*, 2006). The fact that Δ*mep2* mutant was not affected by heat stress, indicated that the absence of this protein does not affect the cell wall, which is compatible with what reasoned above. Nevertheless, the increased sensitivity of Δ*gup1*Δ*mep2* suggests that nitrogen availability and/or Mep2 associated signalling might still be important for the cell to cope with heat stress when the cell wall is affected by the absence of *GUP1*.

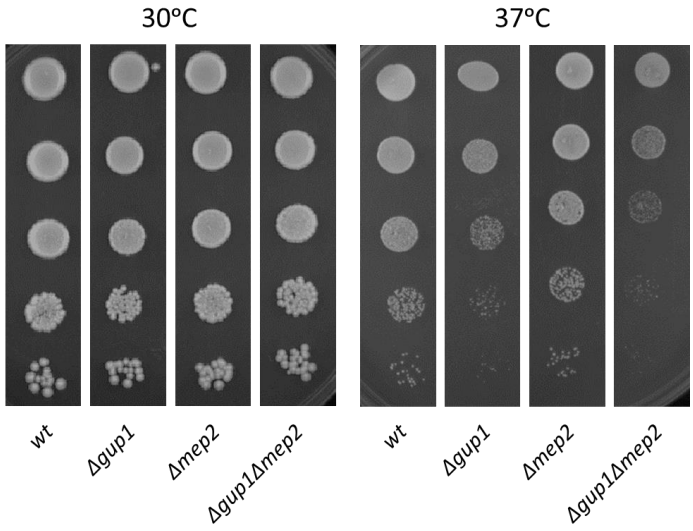

Figure 6 – Effect of high temperature on growth ability *S. cerevisiae* BY4741 *wt, Δgup1, Δmep2* and *Δgup1Δmep2* strains. Strains were grown on YPD until $O.D._{600}$=1 and 10-fold serial dilutions of each culture were spotted onto SLAD medium and incubated for 5 days. One representative experiment is shown

***Cell wall disrupting agents*.** Additionally, cells were also exposed to several well-known wall-perturbing agents: (i) Congo Red (CR) and Calcofluor White (CFW) (Ram and Klis, 2006), which affect cell integrity by binding to chitin and thus interfering with proper cell wall assembly (Roncero and Durán, 1985), and (ii) caffeine, that indirectly activates the CWI pathway through the TORC1 protein kinase complex (Lum *et al.*, 2004; Kuranda *et al.*, 2006). Stressed cells were additionally incubated with sorbitol that is known to remediate growth defects associated with cell wall instability by osmotically stabilizing damaged cells (Levin, 2005). These tests were performed, as

above, in SLAD medium (Fig. 7). As control, the assays were also executed in YPD (not shown).

The phenotypes induced by CR, CFW or caffeine for Δ*gup1* mutant in SLAD medium were similar to those observed in YPD (see Chpt. 3 and 4), and are consistent with a primary defect on cell wall composition and biogenesis (Ferreira *et al.*, 2006). In the presence of CR and CFW, the Δ*mep2* mutant behaved identically to *wt*, suggesting according to above a normal cell wall and functional CWI pathway signalling the response to these stress agents. In opposition, the Δ*mep2* mutant was slightly sensitive to caffeine then *wt* cells (Fig. 7). Caffeine drug activates the CWI pathway indirectly through the inhibition of Tor1-mediated signalling (Kuranda *et al.*, 2006). Mep2 expression and activity are upregulated by the inhibition of Tor1 pathway (Boeckstaens *et al.*, 2014). Thus, it is not unreasonable that the absence of Mep2 causes a phenotype in the presence of caffeine. On the other hand, the double deletion of *MEP2* and *GUP1* slightly increases the sensitivity of yeast to CR, but not to caffeine, whose survival remains similar to that observed in the single deleted mutants. Sorbitol addition as expected, rescued the growth defects in CR and CFW, but not in caffeine.

To our knowledge, Mep2 protein was never associated to the CWI signalling regulation. However, as mentioned before, this protein is regulated by TORC1 pathway (Boeckstaens *et al.*, 2014). Under poor nitrogen conditions, TORC1 pathway is inhibited, which allows the TORC1 effector kinase Npr1 to phosphorylate and activate Mep2 (Boeckstaens *et al.*, 2014). In agreement, rapamycin treatment (a TORC1 inhibitor) promotes Mep2 expression and activation (Hardwick *et al.*, 1999; Boeckstaens *et al.*, 2014). Caffeine treatment was showed to have cellular effects very similar to that of rapamycin, including the increased expression of Mep2 (Kuranda *et al.*, 2006), supporting the idea that TORC1 pathway is the caffeine main cellular target. Interestingly, *GUP1* deleted strain is resistant to rapamycin (Ferreira, 2005). Moreover, *GUP1* expression is significantly reduced after the treatment with rapamycin, being this gene one of the most down-regulated genes after 60 min treatment (Hardwick *et al.*, 1999). This way, inhibition of Tor1 pathway, either by nitrogen depletion, rapamycin or caffeine, increases *MEP2* expression (Hardwick *et al.*, 1999; Kuranda *et al.*, 2006; Boeckstaens *et al.*, 2014), but down regulates *GUP1* expression (Hardwick *et al.*, 1999). These evidences are in agreement with the idea of a putative role of Gup1 as a negative regulator of Mep2, as suggested above.

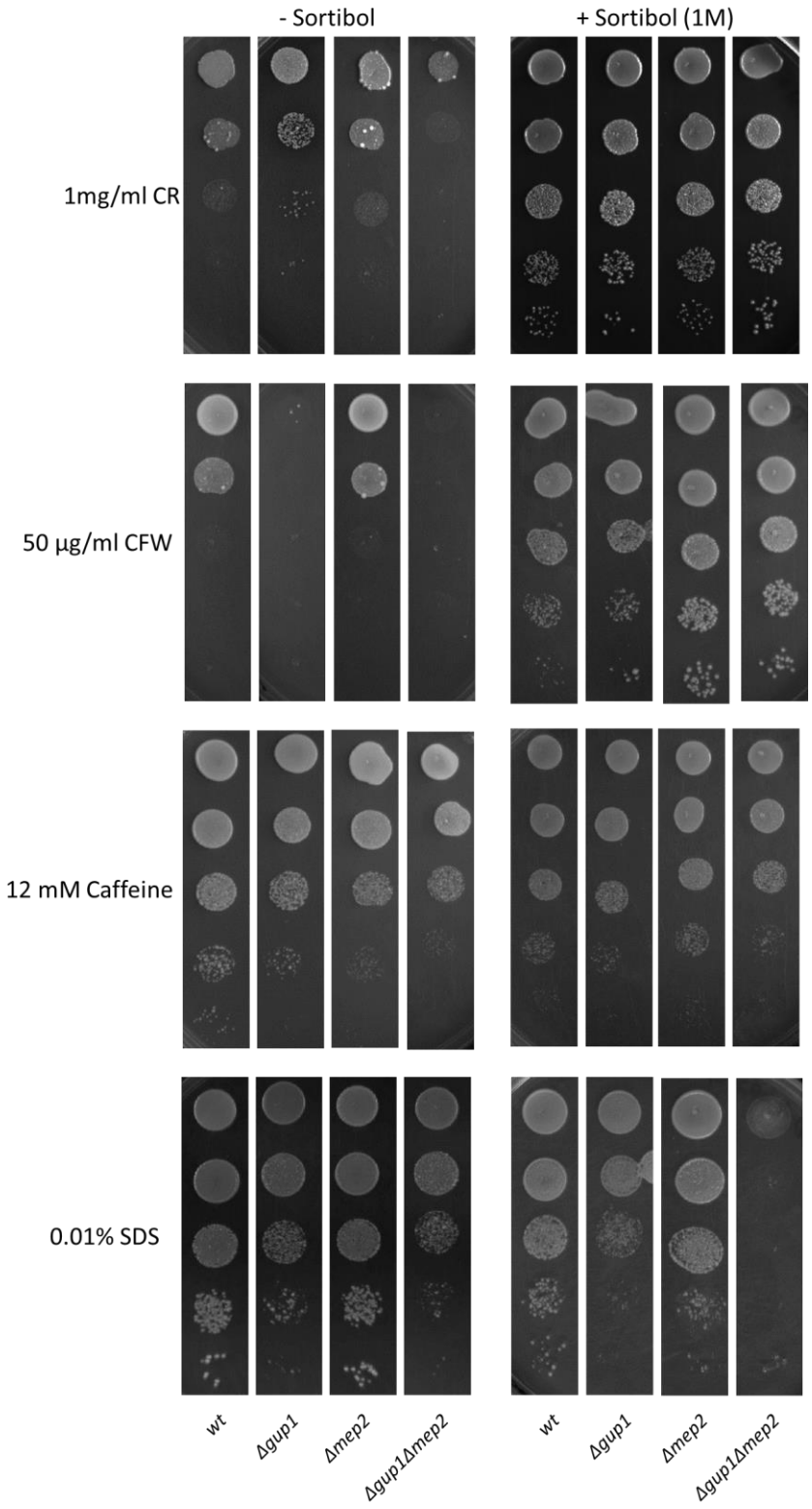

Figure 7 – Membrane and cell wall stress-related phenotypes of *S. cerevisiae* BY4741 *wt, Δgup1, Δmep2* and *Δgup1Δmep2* strains. Cells were grown on YPD until $O.D._{600}$=1 and 10-fold serial dilutions of each culture were spotted onto SLAD media supplemented with different stress agents. Results were scored after a 5 days incubation at 30˚C. One representative experiment is shown.

Plasma membrane and wall disrupting agent. The susceptibility to SDS, a detergent that induces membrane and, indirectly, cell wall stress (Igual *et al.*, 1996), was also evaluated (Fig. 7 – left panels). Unlike Δ*gup1*, Δ*mep2* was not sensitive to 0.01% of SDS, while the double mutant Δ*gup1*Δ*mep2* was slightly more sensitive to the presence of this agent than Δ*gup1*. This sensitivity surprisingly increased when the cells were further incubated with sorbitol (Fig. 7 – right panels), indicating that the cell wall was not the main cellular target of SDS. Instead, SDS was probably causing membrane instability, determining the sensitivity to osmotic stress caused by high concentration of sorbitol on the SDS-weakened cell. Still, this does not justify why the double mutant is more sensitive to SDS and sorbitol than the Δ*gup1*. These results instead support the idea that the deletion of Mep2 in the Δ*gup1* background could result in a pronounced alteration of the plasma membrane organization, structure and/or composition which is already affected in the single Δ*gup1* mutant (Ferreira and Lucas, 2008; Ferreira *et al.*, 2010).

High osmotic stress. Gup1 was long thought to be important for high osmotic stress survival (Hölst *et al.*, 2000; Ferreira *et al.*, 2006), which could indicate a malfunction of the HOG pathway. Consequently, the single and double mutants were cultivated in SLAD medium in the presence of NaCl (1 M), and KCl (1.5 M) (Fig. 8). All these assays were also executed in YPD medium as control (not shown). The effect of 1 M NaCl on SLAD (Fig. 8 – upper panel) was identical for *wt* and Δ*mep2*. On the other hand, the Δ*gup1* and Δ*gup1*Δ*mep2* strains grew identically to each other, and just slightly less than *wt* and Δ*mep2*, which indicates that the deletion of *GUP1* barely affected the survival to 1 M NaCl. The well-known strong phenotype of Δ*gup1* in the presence of 1 M NaCl, that was shown to derive from the inability of the mutant to recover glycerol from the medium (Hölst *et al.*, 2000) due to deficient activity of the Stl1 active permease (Neves *et al.*, 2004; Ferreira *et al.*, 2005), was not observed in cells cultured in the nitrogen-poor medium SLAD. On the other hand, KCl induced a phenotype (Fig. 8 – lower panel) that does not differ from the previous ones of *wt* and Δ*gup1* on YPD (Ferreira *et al.*, 2006; Chpt. 3 and 4), while Δ*mep2* was, identically to NaCl, insensitive to KCl. All taken, the results clearly show that Gup1 function under osmotic stress, which involves the regulation of glycerol uptake (Hölst *et al.*, 2000; Neves *et al.*, 2004), is not dependent of the presence of Mep2.

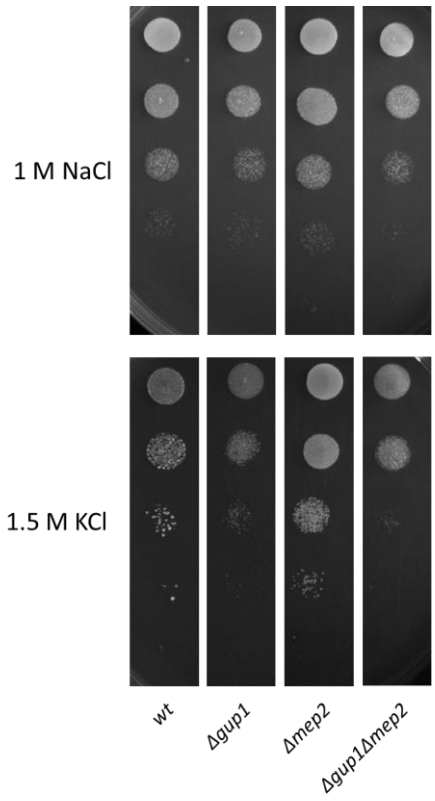

Figure 8 – Osmotic stress phenotypes of *S. cerevisiae* BY4741 *wt*, *Δgup1*, *Δmep2* and *Δgup1Δmep2* strains. Cells were grown on YPD until O.D.$_{600}$=1 and 10-fold serial dilutions of each culture were spotted onto SLAD with 1 M NaCl or 1.5M KCl. Results were scored after a 5 days incubation at 30˚C. One representative experiment is shown.

Conclusions

In a previous work, Van Zeebroeck and colleagues (2011) demonstrated that the membrane-bound *O*-acyltransferase Gup1 is a physical interactor of the ammonium permease Mep2. The same authors showed that the absence of Gup1 causes an increase in the Mep2 transport activity as well as Mep2 associated signalling. In the present work, it was verified that (i) *MEP2* expression level in Δ*gup1* mutant is similar to that measured in *wt* strain, however (ii) Mep2 exhibits an abnormal distribution in the plasma membrane, being more concentrated in some regions, particularly afar form budding site, rather than exhibiting the usual continuous distribution along the plasma membrane. Considering the multiple phenotypes associated with Gup1 that involve

defects in the plasma membrane composition and organisation (Oelkers *et al.*, 2000; Ferreira and Lucas, 2008), and detects in cytoskeleton and bud site selection (Ni and Snyder, 2001; Casamayor and Snyder, 2002), it is fair to assume that the altered Mep2 distribution in the Δ*gup1* mutant might derive from or be associated with them. However, it does not justify the gain of function of Mep2 transporter and signalling when *GUP1* is deleted (Van Zeebroeck *et al.*, 2011). A more direct involvement of Gup1 in Mep2 activity regulation should thus be considered.

In this work we showed that both Mep2 and Gup1 proteins appear to be essential for adherence/invasive growth under low nitrogen conditions, though colony morphology and biofilm-mirroring mat production, did not parallel that requirement. The phenotypic assessment of cellular aspects that were directly or indirectly related to Gup1, and its the interaction with Mep2, also included testing the effect of cell wall stress induced by drugs or culture conditions, including high temperature. Results suggest that those phenotypes are independent of Mep2. Accordingly, so were the cell aggregation and sedimentation phenotypes associated to *GUP1* deletion that were not detected in the Δ*mep2*, but were present in the Δ*gup1*Δ*mep2* cells. Nevertheless, the deletion of *MEP2* in the Δ*gup1* background seems to increase the sensitivity to cell wall stresses, indicating that the Mep2 associated transport and signalling could be important for cell to cope when Gup1 is absence.

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CHAPTER 6

Supplementary Material

Novel results involving Gup2

Gup2 sub-cellular localization

Saccharomyces cerevisiae Gup2 shares a high degree of similarity (77%) and identity (57%) with its close homologue Gup1 (Hölst *et al.*, 2000). These proteins are members of the MBOAT superfamily of multispanning membrane-bound *O*-acyltransferases (Hofmann, 2000), which also comprise their correspondent homologues in higher eukaryotes: HHAT and HHATL. HHAT has more similarity with *ScGUP2*, and is responsible for the Hedgehog-secreted morphogen N-terminal palmitoylation (Chamoun *et al.*, 2001; Buglino and Resh, 2008), whereas HHATL (mammalian Gup1) functions as the negative regulator of the pathway (Abe *et al.*, 2008). In yeast, no marked phenotype was ever observed in association with *GUP2* deletion, neither in response to changes in the carbon source (glucose/glycerol), or to different stress-inducing agents (salts, sorbitol, ethanol, weak acids, high temperature and membrane and wall disturbing agents) (reviewed in Lucas *et al.*, 2016).

Though sequence and conformational predictions suggest that Gup2 is a membrane protein, the determination of the *in vivo* Gup2 sub-cellular localization was assessed for the first time in the present work. *GUP2* gene was amplified from *S. cerevisiae* gDNA by PCR using specific primers:

Fw - 5´GAGAAGCTTATGTCGATGTTAAGAATCTGG3´,

Rv - 5´GAGGGATCCACATTTCAAGTTGATGCCATG3´,

and then ligated in the pYES2-*GFP* vector (Chpt. 2) in order to obtain the construction pYES2-*GUP2-GFP*. This construction was used to transform the W303 Δ*gup2* and Δ*gup1*,*2* mutant strains, complementing the *GUP2* deletion by plasmid expression. The transformation was confirmed by colony PCR. Expression of Gup2-GFP was induced during 6 h by transferring mid-exponential growing cells to YNB medium containing 2% galactose. Protein subcellular localization was monitored by fluorescence microscopy.

In both *wt* and Δ*gup1* strains, the chimera Gup2-GFP seems to be localized mainly in the plasma membrane and endoplasmic reticulum (ER) (Fig. 1). Moreover, the plasma membrane distribution of Gup2 presented a punctate pattern, which indicates a

heterogeneous distribution of this protein, that could *a priori* be consistent with a putative localization at membrane *rafts*. Nevertheless, although Δ*gup1* exhibits a modified plasma membrane organization and integrity, resulting in a different rafts distribution and number (Ferreira and Lucas, 2008), the same localization and distribution of Gup2-GFP was observed in this mutant (Fig. 1). Therefore, the punctate distribution should not derive from the association with rafts.

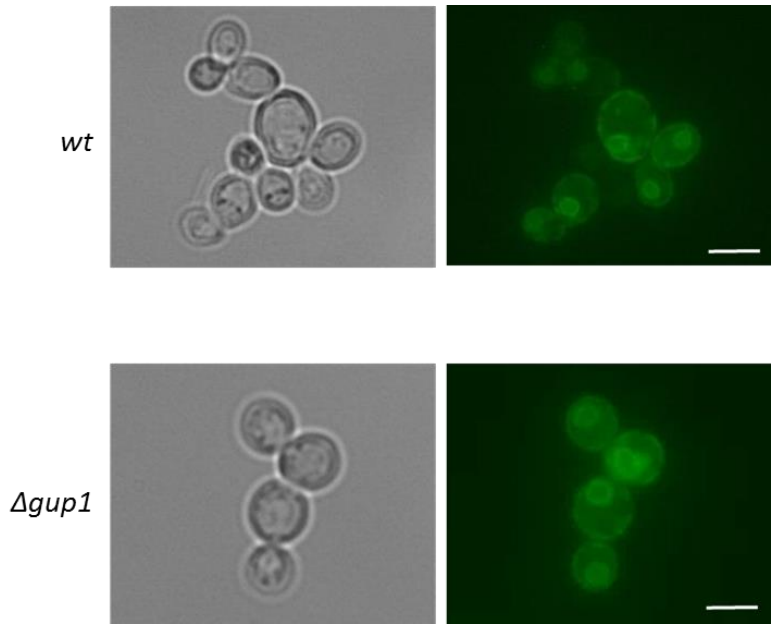

Figure 1 – Fluorescence microscopy visualization of Gup2-GFP localization in *S. cerevisiae wt* and Δ*gup1* strains. Cells carrying the pYES2-*GUP2-GFP* plasmid were grown in YNB glucose medium until mid-exponential phase. Expression of the chimera Gup2-GFP was induced by incubating the cells in YNB galactose medium during 6 h. One representative experiment is shown. Bar = 5μm.

Despite the high degree of similarity between Gup1 and Gup2 (57% identity and 77% similarity), the distribution pattern of Gup1 is different from the now found for Gup2. Although the mainly localization of Gup1 is also the plasma membrane and ER, the distribution of Gup1-GFP is homogeneous (Bleve *et al.*, 2005; this work, Chpt. 2), in contrast to the punctate pattern observed for Gup2-GFP. This could lead to the assumption that the two proteins should have different functions, which agrees with the general absence of *GUP1* deletion-associated phenotypes in the Δ*gup2* mutant strain (Hölst *et al.*, 2000; Ferreira *et al.*, 2006; Ferreira, 2005; Faria-Oliveira, 2013), and with

the fact that these phenotypes were never complemented by *GUP2* overexpression (Ferreira, 2005).

Δ*gup2* mutant cells are sensitive to acetic acid

S. cerevisiae GUP1 disruption induces the hypersensitivity of yeast cells to apoptotic cell death induction by acetic acid (Tulha *et al.*, 2012). Nevertheless, in the presence of lethal concentrations of this compound, *Δgup1,* unlike the wild type, undergoes a necrotic-like cell death process, as suggested by the absence of several apoptotic markers including: (i) preservation of plasma membrane integrity, (ii) phosphatidylserine externalization, (iii) depolarization of mitochondrial membrane, and (iv) chromatin condensation (Tulha *et al.*, 2012). The sensitivity of the Δ*gup2* to acetic acid, as well as the role of Gup2 absence in the previously described Δ*gup1* cell death phenotype, was studied here for the first time. The sensitivity of *wt*, Δ*gup1*, Δ*gup2* and Δ*gup1*Δ*gup2* to acetic acid treatment was determined as commonly assessed to induce apoptotic cell death (Ludovico *et al.*, 2001). Briefly, cells at exponential growth phase were exposed to 150 mM acetic acid during 180 min, and the survival rate measured by c.f.u. quantification (Fig. 2A). Additionally, cells that maintain membrane integrity were measured by PI staining, a typical preliminary assay used to quantify apoptotic cells (Fig. 2B).

As previously described, the Δ*gup1* mutant was more sensitive to acetic acid treatment than *wt* cells, presenting approximately 25% survival in contrast to the 50% survival of *wt* cells (Fig. 2A). Moreover, Δ*gup1* mutant cell death was accompanied by the loss of membrane integrity, as showed by the 65% PI positive cells (Fig. 2B), indicating a non-regulated/necrotic cell death process. The Δ*gup2* and Δ*gup1*Δ*gup2* mutants were also more sensitive than *wt* cells, presenting survival rates similar to that obtained for Δ*gup1* cells. However, in Δ*gup2* mutant strain the number of cells that lost their membrane integrity was significantly lower than in Δ*gup1* treated cells (Fig. 2B), and not coincident with the percentage of cell death (Fig. 2A). Therefore, despite the high sensitivity to acetic acid observed in Δ*gup2* mutant, this strain still exhibits a significant percentage of cells that maintain membrane integrity, suggesting it dies of an apoptotic cell death process, similar to what occurs in the *wt* cells. The double mutant Δ*gup1*Δ*gup2* presented the same results, regarding survival and membrane integrity, observed in the Δ*gup1* single mutant.

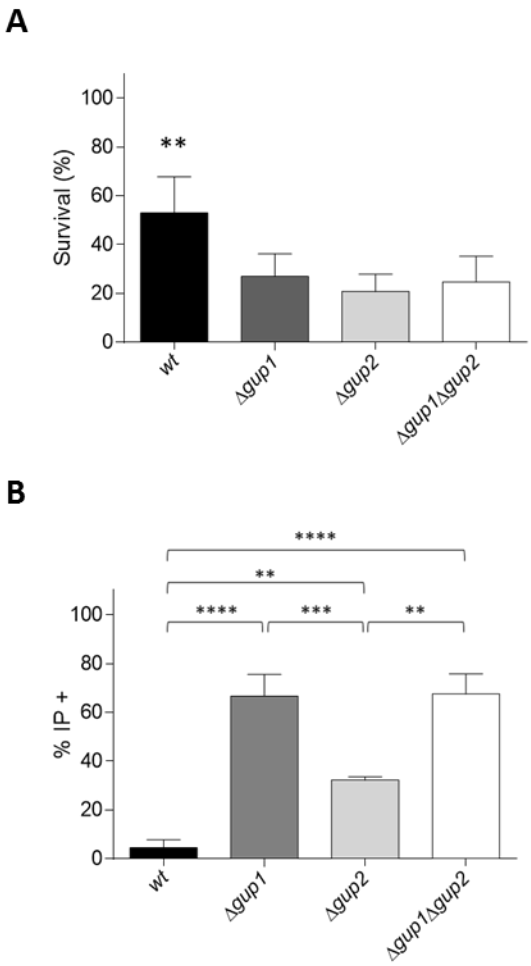

Figure 2 – Cellular response of *S. cerevisiae wt, Δgup1, Δgup2* and *Δgup1Δgup2* strains to acetic acid-induced cell death. Exponentially growing cells were treated with 150 mM acetic acid for 3h. **(A)** Viability was determined by c.f.u. counting (results were normalized with 100% survival corresponding to the total c.f.u. at T_0). **(B)** Graphic representation of the percentage of cells displaying PI staining. Data represent mean ± SD of at least 3 independent experiments. ***P < 0.001; **P < 0.01, one-way ANOVA followed by Tukey's test.

Overall, the results indicate that Gup1 is essential for the preservation of membrane integrity after acetic acid treatment, a process that is independent of Gup2. Furthermore, results also showed that Gup2 might contribute for cell survival upon acetic acid-induced cell death, since its absence lowered the survival rate of Δ*gup2* cells compared to *wt* cells.

Plenty of other phenotypes were associated to the deletion of *GUP1* gene (reviewed in Lucas *et al.*, 2016), however the deletion of *GUP2* does not cause identical responses, neither does it complement the phenotypes of the *GUP1* deleted strain

(Ferreira, 2005). In fact, Δ*gup2* null mutants do not present any marked phenotype in response to osmotic, oxidative, ethanol, temperature or cell wall stress (Ferreira, 2005). Increased sensitivity to acetic acid-induced cell death, observed in the present work, is the first described phenotype that can be clearly associated with the absence of the Gup2 protein. The low responsiveness of the Δ*gup2* mutant made that it was practically abandoned from the research in yeasts, focusing mostly on Gup1. Still, one cannot discard the possibility that Gup2 protein actually has a much more prominent role than thought, if all the functions that underlie the Δ*gup1* mutant phenotypes were performed by an un-inhibited Gup2. This hypothesis became more interesting in view of that mammalian HHATL/Gup1 inhibits the palmitoylation of the Hedgehog secreted morphogen that is ensured by HHAT/Gup2. Evaluating the putative importance of Gup2 in commanding the behaviour of Δ*gup1*, in particular in what concerns a Hedgehog-like cell-to-cell signalling pathway, will require further research.

GUP1 deletion impairs autophagy during starvation

Beside the above-mentioned involvement of Gup1 in acetic acid induced cell death, this protein is also involved in aging. Two different works have suggested that the *GUP1* disruption decreases (Tulha *et al.*, 2012), or extends (Li *et al.*, 2011), yeast chronological life-span (CLS). The main difference between both studies resides in the amino acid availability in the yeast growth medium, which was four times higher in the study by Li *et al.* (Li *et al.*, 2011) than in the one by Tulha *et al.*, (2012). Considering the suggestions that Gup1 might be implicated in or regulated by TOR signalling (reviewed in Lucas *et al.*, 2016), which is controlled by nitrogen/amino acid abundance (Loewith and Hall, 2011), the mutant should respond differently in each cultivation condition. This different behaviour in response to amino acids availability could suggest an involvement of Gup1 in the autophagic process.

Autophagy is the natural, regulated, destructive cellular mechanism that disassembles unnecessary or dysfunctional components. It involves the engulfment of intracellular constituents into double membrane vesicles, named autophagosomes, which then fuse

with the vacuole for degradation of its content (Reggiori and Klionsky, 2013). At basal levels, autophagy is constitutively active, recycling the cell contents to maintain cellular homeostasis and integrity. Additionally, autophagy can be activated in response to starvation and other conditions of metabolic stress, to provide an alternative source of energy that limits cell death (Onodera and Ohsumi, 2005). In mammals, autophagy is also implicated in cancer (Mathew *et al.*, 2007), in innate and adaptive immunity (Levine and Deretic, 2007), and also in cellular development and differentiation (Levine and Klionsky, 2004). The deregulation of autophagy is associated with cell death. Excessive autophagy can lead to an autophagic cell death. On the other hand, deficient autophagy results in the inability of cells to adapt to unfavourable environmental conditions leading to premature death (Abeliovich, 2015). The putative involvement of Gup1 in this process might explain the decrease of Δ*gup1* mutant CLS in low amino acids conditions, due to its inability to recycle nutrients through autophagy.

The autophagy induction after nitrogen starvation in the *GUP* deleted mutants was assessed by monitoring GFP-Atg8 levels. For that, *wt*, Δ*gup1*, Δ*gup2* and Δ*gup1*Δ*gup2* strains were transformed with the pRS416 plasmid carrying the GFP-Atg8 construction under the control of its endogenous promoter (Shintani and Klionsky, 2004) (plasmid kindly provided by S. Alves and M. Côrte-Real from CBMA, University of Minho). Autophagy was induced by nitrogen starving the cells during 24 h (nitrogen starvation media: 0.17% yeast nitrogen base w/o amino acids and ammonium sulphate, supplemented with 2% glucose). The autophagy induction was quantified by measuring the free-GFP in relation to Pgk1 levels by WB. Once autophagy is triggered, Atg8 is normally conjugated to phosphatidylethanolamine on its C terminus, which helps expand the membranes of the autophagosome. The protein GFP-Atg8 is thus transferred to the vacuole through autophagy. If autophagy proceeds normally, Atg8 is degraded while inside the vacuole, but the GFP moiety is resistant to degradation and can therefore be detected as free GFP by WB.

In control non-starvation conditions, no free GFP was detected in any strain. In nitrogen-starved *wt, and* Δ*gup2* strains, GFP-Atg8 processing was detected, suggesting normal autophagy. On the other hand, Δ*gup1* and Δ*gup1*Δ*gup2* strains exhibited extremely reduced levels of autophagy upon nitrogen starvation, as shown by the lower amount of free-GFP in these cells, in contrast to *wt* strain (Fig. 3). This result clearly

demonstrates that autophagy is impaired or at least down-regulated in yeast cells lacking the *GUP1* gene, but not *GUP2*.

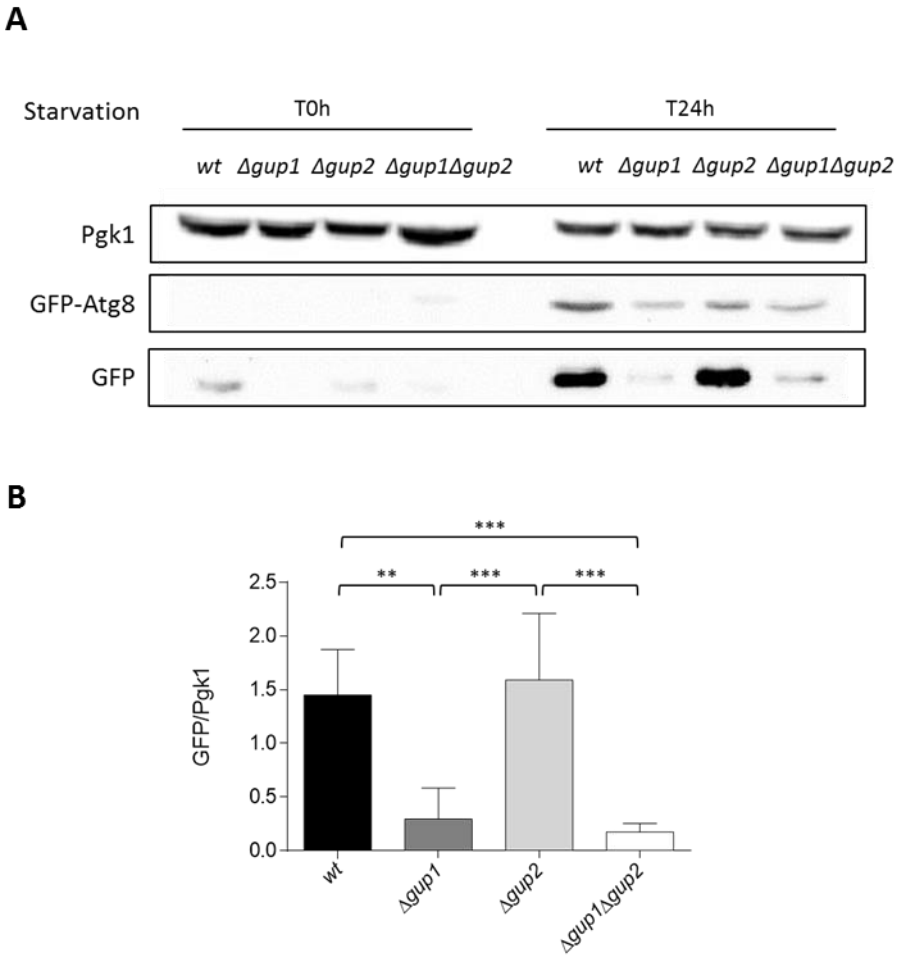

Figure 3 – *GUP1* deletion decreases the processing of GFP-Atg8 during nitrogen starvation. (A) Detection of free GFP generated from the GFP-Atg8 fusion protein, in *S. cerevisiae wt*, Δ*gup1*, Δ*gup2* and Δ*gup1*Δ*gup2* cells expressing GFP-Atg8, after 24h of nitrogen starvation, by immunoblot analysis. Pgk-1 immunoblot was used as loading control. **(B)** Free GFP/Pgk1 ratio after 24 h of nitrogen starvation was determined using ImageJ software. Data represent mean ± SD of at least 3 independent experiments. ***P < 0.001; **P < 0.01, one-way ANOVA followed by Tukey's test.

The regulation of autophagy process is complex. Besides the regulation at the level of the Atg machinery, autophagy is regulated by upstream signalling pathways (Farré and Subramani, 2016). Tor1 pathway has been described as the principal pathway regulating autophagy in yeast, acting as a negative regulator (Kamada *et al.*, 2000; Vlahakis and Powers, 2014; Vlahakis *et al.*, 2014). Still, diverse studies demonstrated that additional signal transduction cascades such as the Ras/cAMP-dependent PKA

pathway cooperate with TOR in the regulation of autophagy (Budovskaya *et al.*, 2004; Wu and Terada, 2010; Alves *et al.*, 2015). TORC1 is primarily involved in regulating nutrient sensing, particularly nitrogen availability, but as aforesaid, TOR is also the major pathway controlling autophagy (Kamada *et al.*, 2000; Staschke *et al.*, 2010; Vlahakis and Powers, 2014; Vlahakis *et al.*, 2014). The promoter regions of both *GUP1* and *GUP2* hold a repeated consensus sequence for Gcn4, a major transcription factor under the control of TORC1 pathway (Valenzuela *et al.*, 2001). Accordingly, *GUP1* gene seems to be depended on Gcn4 for its full expression (Ferreira, 2005), which suggests a possible Gup1 regulation by TORC1. Moreover, the Δ*gup1* mutant, both alone or in combination with Δ*gup2*, is resistant to rapamycin (Ferreira, 2005), a TORC1 pathway inhibitor (Loewith *et al.*, 2002; Jacinto and Lorberg, 2008), which could indicate a malfunction of this signalling pathway in the absence of Gup1. Therefore, the impairment of autophagy after starvation of Δ*gup1* and Δ*gup1*Δ*gup2* mutants could be a consequence of a defective regulation of Tor1 pathway. A possible deficiency at the level of autophagic machinery cannot to be excluded either.

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CHAPTER 7

General Discussion and Future Perspectives

GUP1 is a very pleiotropic gene influencing a great number of seemingly unrelated phenotypic traits. In fact, the deletion of this gene in the yeast *S. cerevisiae* was shown to affect several cellular processes, such as: cell wall and membrane composition and structure, rafts assembly, lipid metabolism, GPI-anchor remodelling, cytoskeleton polarization, endocytic and secretory pathways, vacuole morphology, telomere length, life span and cell death, ECM composition, and the response to several environmental stresses (osmotic, high temperature, oxidative stress, week acids) (Oelkers *et al.*, 2000; Ni and Snyder, 2001; Bonangelino *et al.*, 2002; Casamayor and Snyder, 2002; Askree *et al.*, 2004; Bosson *et al.*, 2006; Ferreira *et al.*, 2006; Ferreira and Lucas, 2008; Tulha *et al.*, 2012; Faria-Oliveira *et al.*, 2015a, 2015b). The disruption of *GUP1* in *C. albicans* also affected several differentiation-associated processes, resulting in altered colony morphology and loss of the capacity to adhere/invade, to differentiate into hyphae, and to form biofilms (Ferreira *et al.*, 2010). Yeast Gup1 and Gup2 proteins are very similar to the mammalian HHATL and HHAT, respectively. HHAT is responsible for the palmitoylation of the Hh morphogen, and HHATL for its negative regulation (Abe *et al.*, 2008; Buglino and Resh, 2008). In high eukaryotes, the Hh pathway is involved with the control of cell differentiation, proliferation and tissue patterning during embryogenesis and wound healing, through the release of a morphogen that transmits a signal from one cell to another. It is, therefore, a form of cell-to-cell communication in which a cell produces a signal to induce changes in the behaviour of nearby cells.

Paracrine signalling has not yet been recognized as occurring in microbial communities. However, the notion that microbial multicellular aggregates can be regarded as *proto-tissues* (reviewed by Shapiro, 1998) predicts that each individual cell does not live exclusively in response to environmental stimuli, as in planktonic life, instead it must involve cell–cell communication as a requirement for group behaviour and survival. In fact, multicellular aggregates of microorganisms, like colonies or biofilms, present differentiated and specialized cells (Donlan, 2002), spatially organized into functional structures (Engelberg *et al.*, 1998; Kuthan *et al.*, 2003) and supported by a complex ECM (Hawser *et al.*, 1998; Ma *et al.*, 2009; Faria-Oliveira *et al.*, 2014). Moreover, they are coordinated by complex communication systems (Miller and Bassler, 2001; Palková and Vachova, 2003). It remains unclear, however, whether this communication may happen through a diffusible chemical, like ammonia (Palková *et*

al., 1997; Palková and Forstová, 2000; Palková and Vachova, 2003), through quorum-sensing chemicals (Chen *et al.*, 2004; Sprague and Winans, 2006), or through a peptide signal like in higher Eukaryotes. The large and diverse proteome found in the yeast ECM (Faria-Oliveira *et al.*, 2015b; Gil-Bona *et al.*, 2015a, 2015b) hampers the easy identification of a putative peptide signal. Concurrently, the high number of proteins identified as being differently excreted in the *GUP1*-deleted mutant ECM compared to the *wt* strain, does not allow the suggestion of a candidate (Faria-Oliveira *et al.*, 2015b). Nevertheless, the fact that unlike Prokaryotes, all Eukaryotes have a Gup/HHAT(L) protein suggests that these proteins participate in some conserved process/mechanism. Moreover, unpublished results from our group, showed that the HHATL from mouse is capable of functionally complement *GUP1* deletion in *C. albicans*, also suggesting a conserved function. One should, however, not discard the hypothesis that proteins from the same group might perform very different roles in different organisms. The plethora of phenotypes from the yeast Δ*gup1* mutant, and the multiple localizations of the Gup1 protein (Hölst *et al.*, 2000), favour the notion that it might have multiple roles.

The work developed under the scope of this thesis aimed to identify the intracellular Gup1 partners, as an indispensable step to unveil the molecular function(s) of this protein and the putative associated signalling cascade(s). The interactome of Gup1 and Gup2 has been assessed by whole genome screenings. One single exception is the ammonium transceptor Mep2 (Van Zeebroeck *et al.*, 2011). Although it has been shown to interact with Gup1, the function that the two proteins might command together has not been identified. This work is the first systematic attempt to identify the Gup1 physical interactions.

Two approaches to found Gup1 partners were tried: (1) expressing *sc*Gup1 in *E. coli* in order to obtain a suitable amount of protein to purify and proceed to affinity chromatography, and (2) using co-immunoprecipitation with Gup1 as bait to "catch" Gup1 partners in native conditions. The first approach was subsequently abandoned, since all attempts to express Gup1 in *E. coli* were unsuccessful. The reasons underlying this are not known, but they match previous equally unsuccessful attempts (Bleve, 2005; Ferreira, 2005). Gup1 is a multisapning membrane protein (Hölst *et al.*, 2000). As mentioned above, it is present in all eukaryotes whose genome was sequenced so far, while absent from prokaryotes (Chpt. 2). This might indicate that this protein is toxic for bacteria, and could thus suffer fast-proteolytic degradation, if translated at all. Co-

immunoprecipitation was therefore optimized, and used to successful identify several Gup1 interacting proteins, form which two novel Gup1 physical interactions were identify: the yeast mitochondrial VDAC (Voltage-Dependent Anion Channel) - Por1, and the eisosome core component Pil1 (Chpt. 2).

Gup1 localizes mostly in the plasma membrane and ER (Hölst *et al.*, 2000; Bleve *et al.*, 2005), but also possibly in the mitochondria (Hölst *et al.*, 2000). This last localization was confirmed in the present work (Chpt. 3). The Gup1 partners identified in this work have different cellular localization: Por1 is a porin located in outer mitochondrial membrane (Blachly-Dyson *et al.*, 1993), while Pil1 is a core component of the eisosomes, localizing therefore at the plasma membrane (Walther *et al.*, 2006). The other Gup1 partner Mep2 shares with Gup1 a plasma membrane localization (Hölst *et al.*, 2000; Bleve *et al.*, 2005). Other putative Gup1 partners identified by HTP surveys locate in the plasma membrane (Fet3), ER (Msc7), vacuole (Vtc4; YHL042W), mitochondria (Sat4), nucleus (Nab2), and cytoplasm (Frk1; Hek2). These different localizations, as mentioned above, could be compatible with Gup1 and its partners having diverse roles.

The interaction between Gup1 and three physical partners, Por1, Pil1 (Chpt. 2) and Mep2 (Van Zeebroeck *et al.*, 2011) was studied. First and foremost, the expression of these proteins, as well as their localization/distribution, was evaluated in the presence and absence of *GUP1* (Chpt. 3, 4 and 5). According to results, the expression of the three Gup1 interacting proteins was not affected by *GUP1* deletion. On the other hand, Por1 and Mep2, had their distribution affected by the absence of Gup1 (Chpt. 3 and 5). Por1 loses the punctate distribution over the mitochondrial membrane, becoming evenly distributed, a phenotype that is not accompanied by an altered mitochondrial morphology (Chpt. 3). Moreover, the cellular level of Por1 is lower in the absence of Gup1, despite a barely unaltered transcription of *POR1*. This could result from increased secretion of Por1 in Δ*gup1* cells, as was previously described by our group (Faria-Oliveira *et al.*, 2015b). The distribution of Mep2 is also affect by the absence of Gup1 (Chpt. 5). In *wt* cells, Mep2 exhibits a granulated but continuous distribution over the plasma membrane (Van Zeebroeck *et al.*, 2011), compatible with the described trimmers association of these transceptors (van den Berg *et al.*, 2016). However, when *GUP1* is deleted, Mep2 loses this even distribution and appears to concentrate away from the budding site, as if it moved towards the apical opposite side of the cell (Chpt.

5). Altered cellular levels and distribution of other proteins that are not physical partners of Gup1 were previously observed in the Δ*gup1* mutant strain. This was the case of the plasma membrane H^+ ATPase Pma1, and the GPI-anchored Gas1 (Ferreira and Lucas, 2008). At least in the case of membrane proteins, their misdistribution in the Δ*gup1* mutant could result from the disrupted assembly and integrity of the ergosterol-rich microdomains (lipid rafts) (Ferreira and Lucas, 2008), and/or the altered lipid membrane composition (Oelkers *et al.*, 2000; Ferreira and Lucas, 2008). Still, in the particular cases of Mep2 and Por1, the picture is probably more complex. In the absence of Gup1, lipid rafts become less stable affecting not only the distribution of membrane proteins, but also of sterol and lipids (Ferreira and Lucas, 2008). When rafts are disrupted, their proteins should shift from a punctate pattern to a homogenous distribution along the plasma membrane, which was not the case of Mep2 as mentioned above. One the other hand, Por1 is localized in the mitochondrial outer membrane, where the presence of rafts is still controversial (Mollinedo, 2012). Thus, the altered rafts distribution cannot straightforwardly be considered the reason underlying the altered distributions of either Mep2 (as suggested by Van Zeebroeck *et al.* (2011)) or Por1. Other phenotypes of the Δ*gup1* strain could contribute to this irregular localization pattern, including the defective secretory pathway (Bonangelino *et al.*, 2002), and the abnormal cytoskeleton polarization and budding site selection (Ni and Snyder, 2001; Casamayor and Snyder, 2002). Finally, despite Pil1 distribution seems not affected in the absence of Gup1, the number of eisosomes is reduced to approximately 50% (Chpt. 4). This will be discussed below in more detail.

Subsequently, the specificity of the interactions between Gup1 and each of the identified partners was assessed. For this purpose, a number of simple assays was chosen bearing in mind several phenotypes previously obtained for Δ*gup1* mutant, and in accordance to the specific cellular roles of each partner. Previous data suggest that yeast Gup1 is, or locates at, a hub between CWI, TORC1, TORC2/YPK, and HOG pathways, being involved in the response to nutrients, stresses and differentiation-related processes (Lucas *et al.*, 2016). Therefore, it is not surprising that *GUP1* deletion-associated phenotypes cover a vast number of pathways controlling basic processes of yeast life, namely associated with growth and development, concurring with the roles of the Gup proteins in Hh pathway in higher Eukaryotes. The phenotypic characterization of Gup1/Partner interaction performed thus included: (i) membrane and cell wall related

stress, (ii) osmotic stress; (iii) cell death in response to acetic acid treatment, (iv) colony morphology related phenotypes, (v) and other considered relevant for a specific partner interaction.

Por1 is a mitochondrial protein mainly involved in the preservation of mitochondrial osmotic stability, regulation of respiration and the control of mitochondrial membrane permeability (Blachly-Dyson *et al.*, 1997; Lee *et al.*, 1998; Sánchez *et al.*, 2001). This last function is crucial in the apoptotic cell death program (Desagher and Martinou, 2000). In *S. cerevisiae*, the absence of either *GUP1* or *POR1* renders cells extremely sensitive to acetic acid-induced cell death, though the Δ*gup1* mutant, contrary to Δ*por1* dies with features of a non-apoptotic cell death (Pereira *et al.*, 2007; Tulha *et al.*, 2012). Interestingly, when both Gup1 and Por1 proteins are simultaneously absent, the sensitivity of the double mutant Δ*gup1*Δ*por1* is almost completely reversed to values identical to those exhibited by *wt* cells (Chpt. 3). This observation indicates that the interaction of these proteins is important for the response to acetic acid-induced cell death, possibly by determining the course of an apoptotic program. In the future, it will be of great interest to further characterize the cell death process in Δ*gup1*Δ*por1* mutant. This should allow to confirm if it truly is a distinct process from that observed in Δ*gup1*, which exhibits traits of necrotic cell death, or if instead the differences derive indirectly from the loss of membrane integrity. This should equally allow to understand if Gup1 acts as a regulator of Por1 during acetic acid-induced yeast cell death, or if this interaction is more related to the regulation of membrane integrity, for instance at the level of sphingolipids metabolism/signalling.

The double deletion of *POR1* and *GUP1* severely compromises growth at 37ºC, on glucose media, but more pronouncedly on non-fermentable carbon sources (Chpt. 3), which might suggest that the mitochondrial function is severely compromised when both proteins are absent. Furthermore, sensitivity to high temperature could also indicate, besides the involvement of mitochondria, the existence of severe phenotypes at the level of cell wall structure and/or biogenesis. In fact, it was verified that the double mutant is extremely sensitive to cell wall disturbing agents, despite the surprisingly resistant phenotype associated to the deletion of *POR1* alone. To our knowledge Por1 was never implicated with the cell wall biogenesis or with cell wall integrity signalling, although it would not be the first time that a mitochondrial protein was associated to cell wall biogenesis. An example is the phosphatidylglycerol phosphate synthase (Pgs1)

(Zhong and Greenberg, 2005; Zhong *et al.*, 2007). It should be interesting to assess the cell wall composition and CWI signalling of the double mutant, or even Δ*por1* cells, and compare it to *wt* and Δ*gup1*.

Additionally, it was observed that the double mutant Δ*gup1*Δ*por1* completely abolishes the formation of complex colonies, which concomitantly exhibited a reduced mat production ability (Chpt. 3). The study of the morphogenic and differentiation related phenotypes associated to this interaction in a more suitable *S. cerevisiae* strain, or even in *C. albicans* whose *GUP1* deletion is implicated in morphogenic and virulence phenotypes (Ferreira *et al.*, 2010), must be assessed in the future.

This work clearly demonstrates, for the first time, the existence of a physical interaction between Gup1 and the mitochondrial protein Por1, adding a new and interesting facet to the cellular processes associated to or in the dependence of Gup1. Although these proteins co-localize in the sub-cellular mitochondrial fraction, the possibility that Gup1 could be interacting with Por1 in the ERMES complex, where Por1 was also found (Stroud *et al.*, 2011), cannot be excluded. The ERMES complexes are involved with the regulation of mitochondrial fission autophagy, and also with the exchange between membranes of ions, proteins and lipids, particularly phospholipids (Michel and Kornmann, 2012). The localization of Gup1 in this structure could underlie the lower amounts of phospholipids in the Δ*gup1* mutant (Oelkers *et al.*, 2000), as well as its impairment of autophagy, reported in this work for the first time (Chpt. 6).

Pil1 protein is a core component of the eisosomes (Walther *et al.*, 2006). Eisosomes are invaginations in the plasma membrane and concentrate several proteins, lipids and signalling molecules (Walther *et al.*, 2006; Strádalová *et al.*, 2009). In the absence of Gup1, as mentioned above, the number of eisosomes is reduced to approximately 50%, which is not associated with a reduction on *PIL1* expression (Chpt. 4). The Pil1 assembly and eisosome formation occurs in the growing buds and is regulated by cell cycle (Moreira *et al.*, 2009). After formation, eisosomes are stable structures, thus the reduced number of eisosomes in the Δ*gup1* mutant could result from a deficient assembly of Pil1 during eisosome formation. To explore this possibility, a cell cycle dependent study must be performed to verified if Gup1 and Pil1 interact in a specific phase of the cell cycle.

Pil1 preferentially binds to the membrane phospholipid PI(4,5)P2 (Karotki *et al.*, 2011). Moreover, Pil1 is crucial for maintaining normal plasma membrane

phosphatidylinositide (PI) levels and availability, by recruiting PI(4,5)P2 phosphatase Inp51 to the plasma membrane (Fröhlich *et al.*, 2014; Kabeche *et al.*, 2015). The levels of this specific phospholipid in the Δ*gup1* mutant are not documented. Still, in the absence of Gup1, cells present a decrease in phospholipids with a concomitant accumulation of diacylglycerols (DAGs) (Oelkers *et al.*, 2000), which are a direct product of PI(4,5)P2 hydrolysis. This suggests that the levels of PI could be reduced in the Δ*gup1* mutant, and could explain the reduced number of eisosomes in Δ*gup1* cells. Therefore, the PI metabolism and the quantification of PI(4,5)P2 must be analysed in the future.

From the results presented in this work, the absence of Pil1 does not provoke any osmotic or cell wall related phenotypes (Chpt. 4). Moreover, the results clearly show that Pil1 does not interfere with the Gup1 effects on osmotic stress and cell wall, which indicate that the interaction Gup1-Pil1 is not important for these Gup1 associated phenotypes. In addition, the deletion of *PIL1* in a Δ*gup1* background, was not able to reverse the necrotic type of death previously observed for this strain (Tulha *et al.*, 2012), which indicates that Gup1-Pil1 interaction is also not relevant for determine the course of cell death. In opposition, the Δ*pil1* mutant was sensitive to SDS, a phenotype that was not remediated by sorbitol (Chpt. 4), which indicates that plasma membrane instability is the primary cause for this susceptibility. The deletion of both *GUP1* and *PIL1* proteins induced an increased sensitivity to this detergent (Chpt. 4), which together with the previously described uniform distribution of ergosterol caused by the absence of each protein by itself (Walther *et al.*, 2006; Grossmann *et al.*, 2007, 2008; Ferreira and Lucas, 2008), supports the idea of important changes on Δ*gup1*Δ*pil1* plasma membrane, possible related to the Gup1-Pil1 interaction. In addition, the loss of PI(4,5)P2 homeostasis in Δ*pil1* mutant (Fröhlich *et al.*, 2014; Kabeche *et al.*, 2015), as well as the altered membrane composition of Δ*gup1* mutant (Oelkers *et al.*, 2000; Ferreira and Lucas, 2008), which could indicate a unbalance of PI levels as mentioned above, support the idea that both proteins are important for plasma membrane integrity, particularly involving the control of PI levels.

The homeostasis of PI(4,5)P2 is set by the balanced activities of PI 5-kinase, which phosphorylates PI 4-phosphate to generate PI(4,5)P2, versus counteracting synaptojanin-related lipid phosphatases, which hydrolyse PI(4,5)P2 to generate PI 4-phosphate and DAG (Strahl and Thorner, 2007). Interestingly, mutation on the PI 5-

kinase reveal phenotypes very similar to those observed for *GUP1* (Lucas *et al.*, 2016). PI(4,5)P2 contributes to a wide variety of cellular process despite its low abundance in the plasma membrane, including the organization of actin polarity and also for cortical actin patches that mediate endocytosis (Desrivières *et al.*, 1998; Homma *et al.*, 1998). Beyond this structural role, PI(4,5)P2 has the ability to control signal transduction particularly through the CWI pathway (Levin, 2011). Therefore, from the list of phenotypes that were associated to Gup1 (reviewed by Lucas *et al.* (2016)), we can speculate that also other effects can derive from Gup1/Pil1 interaction, namely the disruption of the bud-site selection (Ni and Snyder, 2001; Casamayor and Snyder, 2002) and the endocytic pathway (Bonangelino *et al.*, 2002). Also, this interaction might underlie the extensive membrane blebbing observed when Gup1 was overexpressed (Bleve *et al.*, 2011).

Previously, it was demonstrated that Gup1 physically interacts with the ammonium permease Mep2 (Van Zeebroeck *et al.*, 2011). The same authors also verified that the absence of Gup1 causes an increase in Mep2 transport activity as well as Mep2-associated PKA signalling. The nature of the intensification of Mep2 functions is unclear. Still a direct involvement of Gup1 in Mep2 activity regulation has to be considered, possibly involving the negative regulation of Mep2. The deletion of *MEP2* by itself does not promote sensitivity to cell wall stress and high temperatures, or the sedimentation phenotype associated to Δ*gup1* (Chpt. 5). Still, the absence of Mep2 in the Δ*gup1* background seems to increase the sensitivity to cell wall stresses, indicating that the Mep2 function could be important for cells to cope when Gup1 is absent (Chpt. 5). On the other hand, both Δ*gup1* and Δ*mep2* were sensitive to caffeine, a phenotype that was not remediated by sorbitol (Chpt. 5). The double mutant is also sensitive. Caffeine acts similarly to rapamycin, by inhibiting Tor1 pathway. Inhibition of Tor1 pathway, either by nitrogen depletion, rapamycin or caffeine, increases *MEP2* expression (Hardwick *et al.*, 1999; Kuranda *et al.*, 2006; Boeckstaens *et al.*, 2014), but seems to down regulates *GUP1* expression (at least through rapamycin) (Hardwick *et al.*, 1999). Accordingly, these evidences also indicate that Gup1 could negatively regulate Mep2 activity as suggested above.

Mep2 as a transceptor, not only transports ammonium, but also functions as an ammonium sensor, vital for the regulation of filamentous and adherence/invasive growth in both *S. cerevisiae* (Lorenz and Heitman, 1998; Gagiano *et al.*, 1999;

Rutherford *et al.*, 2008) and *C. albicans* (Biswas and Morschhäuser, 2005). The involvement of Gup1 in this process was also previously described in *C. albicans* (Ferreira *et al.*, 2010). Therefore, the involvement of Gup1, as well as the interaction Gup1-Mep2, in this mechanism was analysed. As previously described, Δ*mep2* mutant cells are not able to adhere. This phenotype that was identically found in Δ*gup1* and Δ*gup1*Δ*mep2* (Chpt. 5). In fact, surprisingly, Δ*gup1* mutant was also not able to adhere, despite the associated increased Mep2 transport and PKA signalling (Van Zeebroeck *et al.*, 2011). The mechanism by which Mep2 might sense the ammonium limitation and induce pseudo hyphae growth remains largely unknown. It was previously described that the roles of Mep2 in ammonium transport and induction of filamentous growth could to be separable. Although transport is necessary to induce filamentation and invasive growth it is not sufficient (Biswas and Morschhäuser, 2005; Rutherford *et al.*, 2008). Also, the increased activation of PKA pathway signalling in Δ*gup1* was proposed to be independent of Mep2 function on filamentous growth (Van Nuland *et al.*, 2006). The fact that the double mutant was also incapable of adherence indicates that *GUP1* deletion doesn't complement the loss of adherence described for Δ*mep2* mutant, suggesting that both proteins could have equivalent roles in the invasive/adherence process. Overexpression of Mep2 on Δ*gup1* background, and *vice-versa*, will in the future help to clarify if these proteins interact in the same signalling pathway to control adherence and invasive growth.

Finally, this work also presents novel results involving the Gup1 close homologue Gup2 (Chpt. 6). It was shown that Gup2 is located mainly in the plasma membrane and ER, presenting a punctuated distribution in both *wt* and Δ*gup1* strains, which excludes the association of Gup2 with rafts. Furthermore, results also showed that Gup2 might contribute for cell survival upon acetic acid-induced cell death, since its absence lowered the survival rate of Δ*gup2* cells compared to *wt* cells. Therefore, increased sensitivity to acetic acid-induced cell death, observed here for the first time, is the first described phenotype that can clearly be associated with the absence of the Gup2 protein. Most of the phenotypes caused in yeast by the deletion of *GUP* genes are associated with the *GUP1* deletion. In mammalian and fly cells, the more detailed information available concerning the Gup proteins almost exclusively regards Gup2/HHAT. This functions as the enzyme that performs the palmitoylation of the Hh signal (Buglino and Resh, 2008), while Gup1/HHATL acts as an inhibitor of that palmitoylation (Abe *et al.*,

2008). If that would be the case for yeasts, Gup2 could be performing a role that is inhibited by Gup1 in *wt* cells. Therefore, all the phenotypes associated so far with the deletion of *GUP1* would actually derive from the uninhibited function of Gup2. A detailed work focused in this protein in yeast, as well as the identification of the Gup2 partners will be an extremely important matter of studied in the future, putatively providing valuable information regarding the function(s) of Gup proteins in yeast.

The work developed in this thesis was the first systematic effort to describe the interactome of Gup1 and to understand the biological relevance of these interactions. Three physical partners are hereby study and the interaction characterized: the outer mitochondrial membrane Por1, the eisosome core component Pil1 and the ammonium transceptor Mep2. Altogether, our data undoubtedly show the vital role of the Gup1 partners to the function(s) of Gup1 protein in several cellular processes. The details and extension of these partnerships will have to be assessed in the future, hopefully contributing to clarify of how Gup proteins relate to each other and their partners and how that controls major cellular processes including cell-cell communication in yeast, as happens in higher eukaryotes counterparts HHAT(L).

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