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Mestrado em Genética Molecular

Trabalho realizado sob a orientação de:
Professora Doutora Célia Ferreira
Professora Doutora Cândida Lucas

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DECLARAÇÃO

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Summary

S. cerevisiae GUP1 was initially associated to glycerol uptake through an active system. This, since its deletion caused defects on glycerol-mediated salt-stress recovery and on the glycerol/H^+ uptake V_{max}. Over the last decade, several phenotypes for *Δgup1* strain have been reported, suggesting that Gup1p is involved in a wide range of crucial processes for cell preservation and functioning. These include cytoskeleton polarization and endocytic/secretory pathway, GPI-anchor remodelling, cell wall and membrane composition and integrity, and apoptotic process.

In *Candida albicans*, an opportunistic fungal pathogen, the deletion of this gene interferes crucially on its virulence, preventing the transition to filamentous growth, the ability of adhesion, invasion, and biofilm formation. Moreover, the lack of Gup1p promotes an increased resistance to the common antifungals.

GUP1 is member of the super family of membrane-bound -*O*-acyl transferases, with close homologues in higher eukaryotes. Mammalian *GUP1* has been identified as negative regulator on the Hedgehog signalling pathway. This pathway, in mammals, plays a key role in cell differentiation during embryogenesis, and its malfunction may cause various types of diseases, including cancer. Based on that, the aim of this work concerned the identification of molecular partners of Gup1p on a possible morphogenic pathway in yeast, similar to Hedgehog in mammals.

It was attempted the expression of Gup1 protein in *E. coli* BL21(DE3)-CodonPlus-RIL utilizing the pET expression systems as well as in *S. cerevisiae* null mutant strain using pYES plasmids tagged to a reporter protein GFP and also to a Histidine tag. Despite of the use of high number of conditions and optimizations, the expression of Gup1p in *E. coli* was never attained. On the other hand, using a chimera construction with GFP as reporter gene, the expression in *S. cerevisiae Δgup1* mutant strain was reached and therefore, this system was used in subsequent immunoprecipitation assays, which are still being developed and optimized.

Resumo

O gene *GUP1* de *S. cerevisiae* foi inicialmente associado ao transporte ativo de glicerol. Este gene, quando deletado causa defeitos no transporte do glicerol em situações de stress, assim como na $V_{máx}$ do transporte de glicerol. Durante a última década, foram atribuídos vários fenótipos à estirpe *Δgup1*, sugerindo que a proteína Gup1 está envolvida em diversos processos cruciais para o funcionamento e preservação das células. A proteína Gup1 foi descrita como interferido em processos associados à polarização do citoesqueleto e à composição da membrana e da parede celular, em conexão com as vias de sinalização da secreção e/ou endocitica, à remodelação das âncoras GPI, assim como a processos de apoptose.

Em *Candida albicans*, um fungo patogénico oportunista, a supressão deste gene interfere crucialmente na sua virulência, impedindo a transição para o crescimento filamentoso, interferindo na capacidade de adesão e de invasão, e na formação de biofilmes. Além disso, a deleção do gene *GUP1* aumenta a resistência aos antifúngicos comuns.

O gene *GUP1* é membro da família das *O*-aciltransferases, com homólogos próximos em eucariotas superiores. O gene *GUP1* em mamíferos foi identificado como regulador negativo da via de sinalização Hedgehog. Esta via, nos mamíferos desempenha um papel fundamental na diferenciação celular durante a embriogénese, e o seu mau funcionamento pode causar vários tipos de doenças, incluindo o cancro. Tendo isto em conta, o objetivo do presente trabalho pretendia identificar os possíveis parceiros moleculares da proteína Gup1, numa eventual via morfogénica de leveduras, semelhante à via Hedgehog dos mamíferos.

A expressão da proteína Gup1 em *S. cerevisiae Δgup1* foi testada usando os plasmídeos pYES ligados à proteína repórter GFP e a uma cauda de histidinas, assim como em *E. coli* BL21(DE3)CodonPlus-RIL utilizando os plasmídeos pET. Foram experimentadas diferentes condições para obter a expressão da proteína Gup1 em *E. coli*, contudo não se obteve expressão. Por outro lado, a expressão da proteína Gup1 em *S. cerevisiae Δgup1* foi obtida a partir da quimera *GUP1-GFP*. E como tal, este sistema foi utilizado nos ensaios subsequentes de imunoprecipitação, que se encontram presentemente em fase de desenvolvimento e otimização.

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Introduction

1. *Saccharomyces cerevisiae GUP1*

GUP1 and its homologue *GUP2* were first described to be involved into the active uptake of glycerol (Holst, *et al*., 2000). This, was based on the fact that the deletion of *GUP1* resulted: on a slow growth on glycerol -based media (sole carbon source), on a diminished ability to be rescue from osmotic stress in the presence of extracellular glycerol, and importantly also caused a reduction on the glycerol active uptake V_{max}. The *GUP2* gene, in turn was obtained based on the high molecular homology between the *GUP1* and *GUP2*, these genes have 57% degree of identity and 77% similarity. Despite of the degree of similarity displayed, the authors found that deletion of *GUP2* gene did not present similar phenotypes to *GUP1* and did not complement Δ*gup1* mutation (Holst *et al*., 2000).

Gup1p is predicted to have 12 transmembrane domains and based on sequence and function homology studies is a member of the super family of membrane bound *O*-acyltransferases, suggesting a function as a diacylglycerol *O*-acyltransferase or a closely related one (Ferreira, *et al.*, 2006; Hofmann, 2000; Neves, *et al.*, 2004; Bleve, *et al.*, 2005). Subcellular localization of the Gup1p seems to be quite broad, and was investigated by two different experiments. First, Holst and collaborators showed that Gup1p had a multi- subcellular localization, it was present at the plasma membrane, also in the endoplasmic reticulum and mitochondrial membrane, based on subcellular fractionation by sucrose gradient and detected by immunofluorescence (Holst, *et al.*, 2000). On the other hand, Bleve and co-workers localized Gup1p mainly at the plasma membrane and at the endoplasmic reticulum level, by immunoelectron microscopy and by confocal laser scanning microscopy detection of the chimera GFP-*GUP1* (Fig. 1) (Bleve, *et al.*, 2005). In addition, the authors also defined the Gup1p topology: the N-terminus exists in periplasmic space whereas C-terminal end has an intracellular location (Bleve, *et al.*, 2005).

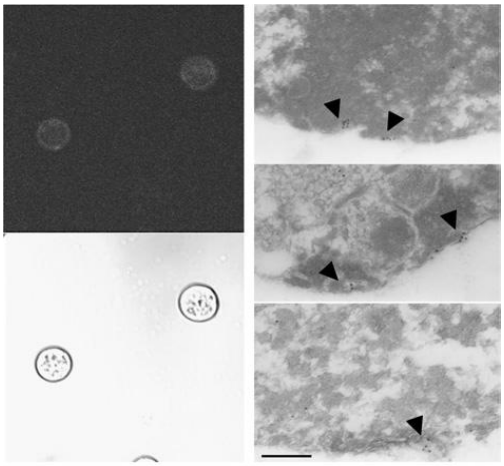

Figure 1 Subcellular localization of Gup1-GFP by confocal laser scanning microscopy (A) and by Immunoelectron microscopy detection (B) (Bleve, *et al.,* 2005).

In their natural habitats, yeasts are commonly exposed to highly variable changes in their osmolarity, but also in the quality/accessibility of nutrients, in temperature, pH, radiation, oxygen availability, as well as in the water activity fluctuations (Blomberg and Adler, 1992). One of the strategies a cell undertakes to survive a sudden environment change in hiper-osmotic pressure is the production and intracellular accumulation of osmolytes, such as glycerol, arabitol, mannitol, and erythritol (Brown and Simpson, 1972; Brown, 1974; Yancey, *et al.,* 1982; van Eck *et al.* 1993; Lages and Lucas, 1997; 1999; Ferreira, *et al.,* 2005). Yeast osmoadaptation mechanism, involves the accumulation of glycerol in the cytoplasm up to molar concentrations, which counteracts cell dehydration and turgor (reviewed by Hohmann, 2002). In *S. cerevisiae* glycerol is synthesized via a short branch of glycolysis, consisting of two-reaction process (Ansell, *et al.,* 1997; Norbeck, *et al.,* 1996). Glycerol is produced from the glycolitic intermediate dihidroxyacetone phosphate catalyzed by NAD^+-dependent glycerol 3-phosphate dehydrogenase and glycerol-3-phosphatase – encoded by two similar isogenes, *GPD1* and *GPD2*, and *GPP1* plus *GPP2*, respectively (Larsson, *et al.,* 1993; Albertyn, *et al.,* 1994; Eriksson, *et al.,* 1995; Norbeck, *et al.,* 1996). Theenzymes for glycerol assimilation are located at the mitochondria: glycerol kinase encoded by *GUT1* and a specific flavin adenine dinucleotide (FAD)-dependent glycerol-3-phosphate dehydrogenase (GPDH) encoded by *GUT2* (Rønnow and Kielland-Brandt, 1993; Sprague, *et al.,* 1977). Mutants defective in *GUT1* or *GUT2* are unable to utilize glycerol as sole carbon source (Rønnow and Kielland-Brandt,

1993, Sprague, *et al.,* 1977). Expression of *GPD1* and *GPP2* increases in cells exposed to a high external osmolarity, which causes a raise in the production, and consequently intracellular accumulation, of glycerol (Andre, *et al.,* 1991, Albertyn, *et al.,* 1994; Eriksson, *et al.,* 1995). On the other hand, *GDP2* and *GPP1* are not affected by changes in external osmolarity, but are stimulated by anoxic conditions (Ansell, *et al.,* 1997). Osmotic induction of *GPD1* and *GPP2* expression is controlled partly by the High Osmolarity Glycerol (HOG) pathway (reviewed by Hohmann, 2002). The inability of mutants with an inactive HOG pathway to adapt properly to high-osmolarity conditions, and the discover of the function of several genes, whose expression is stimulated via the HOG pathway, contributed to the acknowledgment that the cellular role of the HOG pathway is indeed to orchestrate a significant part of the transcriptional response of yeast cells to high osmolarity. The HOG pathway also mediates post-transcriptional effects (Hohmann, 2002). Another MAP kinase pathway, the Cell Integrity Pathway (PKC), can be as well stimulated by changes on osmotic pressure, though by hypo-osmotic shock. Yet, PKC pathway does not appear to be involved in the control of *GPD1* expression (Rep, *et al.,* 1999).

1.1. Glycerol transport across the membrane

The *FPS1* gene was firstly described as a suppressor of the growth defect of the *fdp1* mutant causing inability to grow on glucose-based medium (van Aelst, *et al.,* 1991). *FPS1* gene encodes a protein belonging to the Major Intrinsic Family (MIP) of channel proteins, and has been shown to affect the transport of glycerol across the membrane of *S. cereviasiae* (Luyten, *et al.,* 1995; Sutherland, *et al.,* 1997; Tamás, *et al.,* 1999). Cells lacking Fps1p show a flux of glycerol, both into and out of the cell, highly diminished, and display a number of phenotypes consistent with an inability to rapidly export glycerol from the cell (Luyten, *et al.,* 1995). Since Fps1p is a glycerol channel necessary under specific conditions, it is strongly regulated. The Fps1p closes in response to hyper-osmotic shock to prevent glycerol leakage and retained glycerol within the cell (Luyten, *et al.,* 1995). In contrast, the Fps1p channel opens upon a hypo-osmotic shock, which results in rapid and massive glycerol efflux (Tamás, *et al.,* 1999). At the same time the high osmolarity glycerol (HOG) signal transduction system is activated (de Nadal, *et al.,* 2002; Hohmann, 2002). One of the responses

mediated by the active Hog1 protein kinase is the stimulation of glycolysis, in order to enhance the production of the osmolyte glycerol (Dihazi, *et al.*, 2004).

A third process to regulate intracellular accumulation of glycerol under osmotic stress occurs through an active glycerol uptake system. Lages and Lucas have shown the presence of two mediated transport systems for glycerol in *S. cerevisiae*, depending on the growth conditions (Lages and Lucas 1997). In the absence of salt/osmotic stress or turgor change, glycerol comes into the cells by facilitated transport, through the glycerol channel, encoded by *FPS1* (van Aelst, *et al.*, 1991; Luyten, *et al.*, 1995; Sutherland, *et al.*, 1997; Tamás, *et al.*, 1999; Oliveira, *et al.*, 2003). However, under gluconeogenisis conditions, *e.g.* presence of glycerol, ethanol, acetate, etc, a proton symporter system ensures uptake of glycerol from the environment against concentration gradient (Lages and Lucas, 1997; Sutherland, *et al.*, 1997).

The activity of glycerol active transport has been shown to be essential under salt-stress survival (Oliveira, *et al.*, 1996). As mentioned before, Gup1p was initially suggested as the glycerol transporter (Holst, *et al.*, 2000). However, latter on, the structural gene encoding the active glycerol/H^+ transporter was identified and fully characterized, *STL1* (Ferreira, *et al.*, 2005). This it had been initially ascribed to the HXT family of sugar transporters (Nelissen, *et al.*, 1997). *STL1* expression is regulated in a complex manner, repressed by glucose, induced by growth in gluconeogenic conditions and by salt and osmotic shock in a Hog1p and Hot1p dependent manner (Lages and Lucas 1997; Rep, *et al.*, 2000; Gasch, *et al.*, 2000; Ferreira, *et al.*, 2005). Moreover, Stl1p has been shown as well to respond to other type of stresses, namely low temperature stress (Tulha, *et al.*, 2010) and high temperature stress, situation in which the glucose repression is reverted (Ferreira and Lucas, 2007). The identification of the transporter was supported by several points of evidence (i) *stl1* mutants are not able to efficiently utilize glycerol as sole carbon and energy source, (ii) in *stl1* mutants the active uptake of glycerol is absent, (iii) there is a full correlation between *STL1* expression and glycerol uptake activity, and (iv) the proper functional localization at the plasma membrane (Ferreira, *et al.*, 2005). This data strongly indicated that *GUP1* and *GUP2* did not encode the active glycerol/H^+ transporters, but, instead these genes might have some other function/s, which would indirectly interfere with the glycerol active transport activity.

1.2. Pleitropic function of Gup1p

Besides the mentioned interference on glycerol active transport and defective growth on non-fermentable carbon sources, other reports pointed for a more complex function of Gup1p in yeast cellular metabolism, since the deletion of *GUP1* influenced several cell biogenesis and structure/organization processes. *GUP1* has been described as having a role in vacuolar protein sorting (Bonangelino, *et al.,* 2002), also appears to be involved in bipolar selection and cytoskeleton polarization (Ni and Snyder, 2001), in the telomere length maintenance (Askree, *et al.,* 2004), in GPI-anchor remodelling (Bosson, *et al.,* 2006; Jaqueneoud, *et al.,* 2008), as well as on the cell wall composition and functionality (Ferreira, *et al.,* 2006), lipid metabolism and plasma membrane composition, assembly and integrity (Ferreira and Lucas, 2008). . *GUP1* was also shown to be essential for anaerobic sterol uptake (Ferreira and Lucas, 2008) and plays a crucial role in the apoptotic process in yeasts (Tulha, *et al.,* 2012).

Bonangelino and collaborators screened a collection of *S. cerevisiae* deleted strains, and showed that Δ*gup1* mutant presented aberrant vacuole morphology (Bonangelino, *et al.,* 2002). Indeed, these studies in yeast are particularly important to understand lysosome biogenesis in mammals, as vacuole protein sorting mechanism is highly conserved between yeast and higher eukaryotes (Bonangelino, *et al.,* 2002). Other important phenotype of Δ*gup1* mutant strain was obtained by Ni and Snyder. These authors also screened a collection of homozygous diploid yeast strain deletions in order to identify nonessential genes associated with bipolar budding pattern, having found that *GUP1* was one of those genes (Ni and Snyder, 2001). Polarized cell division is a fundamental process for the development of both eukaryotes and prokaryotes through which cells divide along specific cleavage plans.

Other evidence suggesting the pleitropic nature of Gup1p is its involvement with lipid membrane composition (Oelkers, *et al.,* 2000), functionality, assembly and integrity (Ferreira, *et al.,* 2006; Ferreira and Lucas, 2008). The Δ*gup1* mutant strain has a reduced phospholipid synthesis and increased triacylglyceride synthesis (Oelkers, *et al.,* 2000). Regarding plasma membrane functionality Ferreira and collaborators showed an increased sensitivity of the Δ*gup1* mutant strain to ethanol and weak carboxylic acids suggesting a malfunctioning membrane potential (Ferreira, *et al.,* 2006). In what concerns the plasma membrane assembly, it has been shown that this

mutant presented an even distribution of sphingolipids sterol ordered domains (commonly known as lipid rafts), in contrast with the normal punctuated distribution displayed by the wild type (Ferreira and Lucas, 2008). The authors extract the detergent resistant membrane (DRM) fractions, which contains those ordered domains and analysed the distribution of sterols stained with filipin in the Δ*gup1* mutant plasma membrane by microscopy fluorescence, having found as mentioned a lower number of aggregates in the mutant, (Fig. 2) (Ferreira and Lucas, 2008). Moreover, the activity of H^+-ATPase Pma1p (a plasma membrane protein localized at the sphingolipids sterol ordered domains) was indirectly analyzed by recording the velocity of extracellular acidification. The Δ*gup1* mutant cells showed a reduction of about 40% in the Pma1p-derived external acidification capacity. In addition, Pma1p was found in lower amounts in the total cell extracts of Δ*gup1* mutant than the wild type (Ferreira and Lucas, 2008). Being the Pmap proton pump the main responsible for the maintenance of p.m.f (proton motive force) across the plasma membrane, this data provided the first explanation for the indirect involvement of *GUP1* on the glycerol symporter activity observed, precisely a reduction of about 40% on the V_{max} (Holst, *et al.*, 2000, Ferreira and Lucas, 2008).

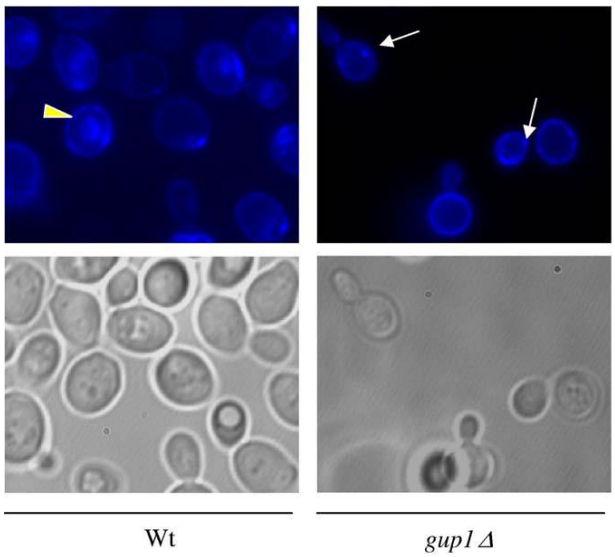

Figure 2 *GUP1* mutation results in a defective sterol lipid distribution. The images show filipin staining of the wt and *gup1Δ* mutant cells (Ferreira and Lucas, 2008).

In addition, ergosterol modulates membrane fluidity, permeability and the activities of membrane-bound enzymes (Smriti, *et al.*, 1999; Parks, *et al.*, 1995). Ergosterol is as

well the target of common antifungals like azoles and polyenes (Pasrija, *et al.,* 2005). Ferreira and Lucas showed that *S. cerevisiae* cell lacking *GUP1* gene presented a high resistance to ergosterol biosynthesis inhibitors (EBI's), including azoles; and in contrast these cells had moderate sensitivity to sphingolipids biosynthesis inhibitors (SBI's), consistent with an involvement of this gene on the cellular lipid metabolism (Ferreira and Lucas, 2008).

Gup1p was shown to further interfere with the cell periphery, namely with the cell wall (Ferreira, *et al.,* 2006). Δ*gup1* mutant strain is sensitive to cell-wall perturbing agents, such as Calcofluor White, Zymoliase and Sodium Dodecyl Sulphate (SDS), and exhibits a sedimentation/aggregation phenotype (Ferreira, *et al.,* 2006). Consistently to those phenotypes, the authors showed that Δ*gup1* mutant presented an altered cell wall composition, with increasing amounts of β-1,3-glucans and chitin and decreasing content on mannoproteins. The reduction on mannoproteins has a direct consequence on an increased permeability of the cell wall, which is consistent with the general pattern of sensitivity to salt, ethanol and carboxylic acid, as well as to the other cell wall perturbing agents (Ferreira, *et al.,* 2006).

More recently, it was reported that Gup1p is required for several cellular processes that are related to apoptosis development (Tulha, *et al.,* 2012). *GUP1* defective strain was shown to have significantly reduced chronological lifespan as compared to wild type (Wt). The authors showed that Wt and Δ*gup1* mutant strains have a notorious differences on apoptotic markers, namely on the phosphatidylserine externalization, on the depolarization of mitochondrial membrane, on the chromatin condensation and on the maintenance of plasma membrane integrity (the latter consistent with the previous results). In addition, it presents extremely high levels of ROS (reactive oxygen species - component of the response of immune cells) and it is also highly sensitive to acetic acid treatment. That data suggested that the *gup1Δ* mutant strains appeared to be experiencing a necrotic cell death process and not an apoptosis event. Furthermore, that study revealed for the first time, the connection of yeast lipid rafts and apoptosis induction and/or signalling, similarly to what happens in mammals, giving new insights into the molecular mechanisms underlying this process in yeast (Tulha, *et al.,* 2012).

2. *Candida albicans GUP1*

In *C. albicans* the deletion of *GUP1* gene causes morphologic and physiologic alterations comprising several virulence factors, namely filamentous growth defects, resistance to antifungal agents, inability of the cells to adhere, invade and form biofilms, as well as colony morphology/differentiation defects (Ferreira et al, 2010). All these phenotypes can be correlated to the virulence of *Candida* species.

2.1. *Candida albicans*

The dimorphic yeast *Candida albicans* is one of the most persuasive fungal pathogens in humans (Odds, 1985). This fungus is an opportunistic pathogen responsible for several infections and diseases. Its success as a commensal and pathogen, and its capacity to invade virtually every tissue, generate biofilms, escape the immune system, resist antifungal treatment and immunomodulate the host defences, suggests a high degree of phenotypic and genomic plasticity and adaptability (Chaffin, *et al.*, 1998; Soll, 2003; Sudbery, *et al.*, 2011). An example is the appearance of an isochromossoma V that carries genes involved on the expression of ergosterol pathway enzyme targeted by azoles drugs (Selmecki, *et al.*, 2006).

The pathogenicity/virulence of *C. albicans* depends on large extent on an effective battery of putative virulence factors and specific strategies that this organism have developed to assist in their ability to colonize host tissues and cause disease (Naglick, *et al.* 2003), including dimorphic growth, adherence ability, invasion ability, capacity to form biofilms and to overcome host defences (Naglick, *et al.*, 2003). Such virulence attributes fall into three categories including, host recognition by fungal cell surface adhesins, the secretion of putative invasive biomolecules such a proteases and phospholipases and morphogenic conversion from a unicellular growth form (yeast) to a filamentous form.

2.2. Morphogenesis

The ability of *C. albicans* to switch spontaneously and reversibly from yeast form growth to *hyphal* growth provides critical functions for the pathogenic lifestyle (Sudbery, *et al.*, 2004). In fact, *C. albicans* has the ability to grow with three distinct

morphologies – yeast, pseudo-*hyphae*, and true *hyphae* (Fig. 3). In the yeast form (Fig. 3a), cells growth by budding-off daughter cells that typically disassociate from the mother cell. In pseudo-*hyphal* cells (Fig. 3e) the buds elongate and fail to separate from the mother cell producing filaments of elongated buds retaining constrictions at the septal junctions. Pseudo-*hyphal* filaments (Fig. 3f) consist of cells which are so elongated that they resemble *hyphae*. In addition, this fungus can naturally occur in other morphological forms characteristic of specific cellular functions, such as *chlamydospore form* – large cells with thick wall and high lipids and carbohydrates content that growth in environments with low oxygen, light, temperature and nutrients (Fabry, *et al.*, 2003; Whiteway, *et al.*, 2007).

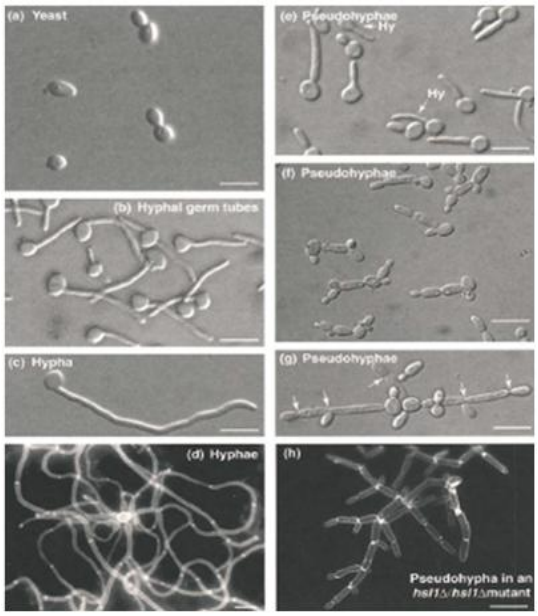

Figure 3 Yeast, hyphal and pseudohyphal morphologies. (a) Unicellular yeast similar to diploid *Saccharomyces cerevisiae;* (b) formation of tubes that will give rise to (c) (d) hyphae In the formation of pseudohyphae the daughter cell elongates and after the formation of the septum remains attached to the mother cell, (f) (g) (h) forming constrictions in the septum (Sudbery, *et al.*, 2004).

In order to investigate if *CaGUP1* was implicated in C. *albicans* morphogenesis, Ferreira and collaborators examined the *hyphal* growth of the corresponding mutated strain, both in liquid and solid media (Ferreira, *et al.*, 2010). *Cagup1*Δ null mutant in liquid media under filamentous growth inducing conditions showed an immense delay to develop filamentous cells, of about 4-5hours (Fig. 4A). On the other hand, and in opposition to wild type, the colonies of *Cagup1*Δ null mutant strain did not show filaments, either peripheral or inside the colony, suggesting that the mutant lost the

ability to form *hyphae*. Furthermore these colonies show remarkable distinct/aberrant morphology when compared to Wt (Fig. 4) (Ferreira, *et al.*, 2010).

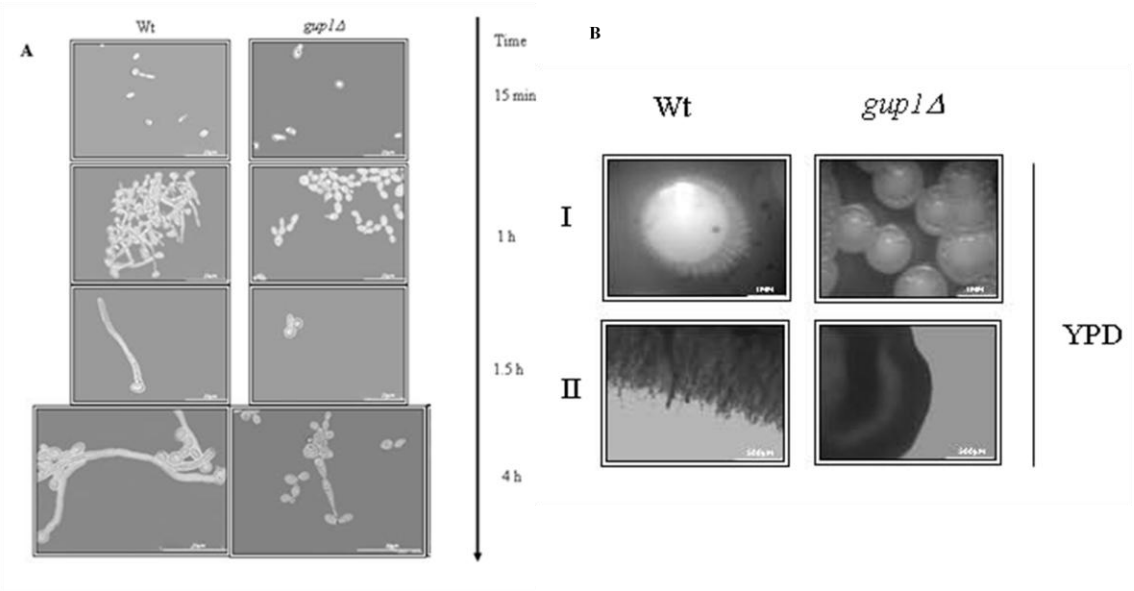

Figure 4 (A) Samples of *Cagup1Δ* null mutant strain and Wt at increasing time points by light microscope. (B) *Cagup1Δ* null mutation leads to aberrant colony morphology, precluding filamentous growth. *Cagup1Δ* null mutant strain colonies are wrinkled (spaghetti/flower shaped) with no peripheral filamentous growth (I). The contour of these colonies observed with light microscopy, fully confirms this absence (II) (Ferreira, *et al.*, 2010).

2.3. Antifungal drugs resistance

Fungal cells, like other organisms become resistant to toxic compounds (Bosshe, *et al.*, 1997). The widespread and prolonged usage of drugs to combat human candidiasis, in recent years has led to the rapid development of the phenomenon of resistance, which poses a major threat to antifungal therapy (White, *et al.*, 2002). There are four groups of drugs available to treat infections. These antifungal agents inhibit macromolecule synthesis interfering with DNA and RNA synthesis (flucytosine) (Bossche, *et al.*, 1997; Pfaller, *et al.*, 2012), damage membrane barrier function (polyenes), inhibit ergosterol synthesis (allylamines, thiocarbamates, azole derivatives, morpholines) or interact with microtubules (griseofulvin) (Bossche, *et al.*, 1997). As azoles, the most common used drugs are fungistatic, rather than fungicides, *C. albicans* cells repetitively exposed to these antifungals can adapt to the drug pressure and eventually become resistant to azoles. Ferreira and co-workers

examined the growth performance of some clones of Ca*gup1Δ* null mutant in the presence of some common antifungals and compare them with Wt. They concluded that the deletion of *C.albicans GUP1* promotes resistance to common antifungals (Fig. 5) (Ferreira, *et al.*, 2010). The same resistance had been previously observed for *S.cerevisiae GUP1* defective strain. Furthermore, and again in accordance with the results with *S. cerevisiae* strain; the deletion of *GUP1* gene in *C.albicans* provokes an aberrant ergosterol distribution, at the level of plasma membrane (Ferreira and Lucas, 2008).

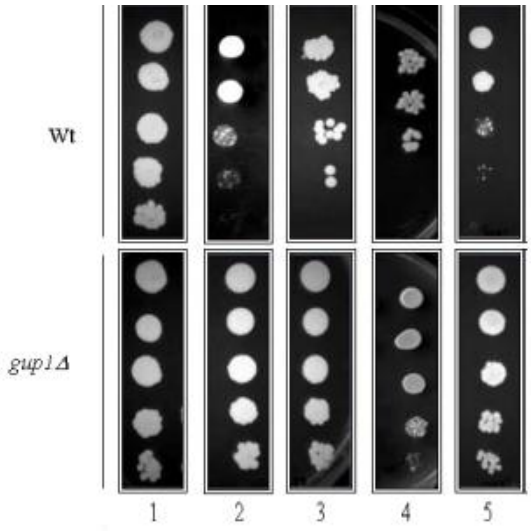

Figure 5 *Cagup1Δ* null mutant strain displays an altered sensitivity to specific ergosterol biosynthesis inhibitors. Isogenic wt, *Cagup1Δ* null mutant strain were spttoted onto (1) YPD plates (control) and plates supplemented with (2) clotrimazole 68.8 μg/ml, (3) ketoconazole 106.3 μg/ml, (4) fluconazole 30.6 μg/ml and (5) fenpropimorph 60 μg/ml (Ferreira, *et al.*, 2010).

2.4. Extracellular matrix and Biofilms in *Candida albicans*

One of the most contributions to *Candida* virulence is its versality in adapting to a variety of different habitats and ability to form surface-attached microbial communities, known as biofilms (Costerton, *et al.*, 1995). Biofilms are aggregates of microbial communities and embedded in an extracellular polymeric matrix – a complex mixture of substances secreted by cells of the biofilm composed of polysaccharides, proteins, nucleic acids and metabolites (Donlan, *et al.*, 2001; Nett, *et al.*, 2006; Beauvais, *et al.*, 2009). Consistently, observations by Scanning Electron Microscopy (Fig. 6) reveal that a fully mature biofilm of *C.albicans* typically consist of a dense network of yeast cells, *hyphae* and pseudo-*hyphae*, within ramifying water channels entrapped on the extracellular polymeric material (Hawser, *et al.*, 1994).

They exhibit a rather complex three-dimensional architecture, likely indicative of a high degree of specialization reminiscent of what is found in primitive mammal tissues (Uppuluri, *et al.*, 2009).

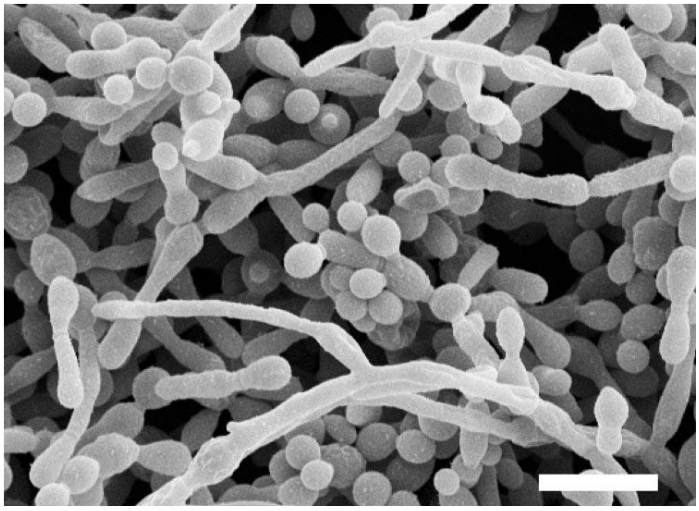

Figure 6 SEM image of a mature (48 h) *C. albicans* biofilm. Note that biofilms are composed of yeast, hyphal, and pseudohyphal elements. Bar is 10 _m (Ramage, *et al.*, 2005).

As mentioned before, the ability of *C.albicans* to exist in more than one morphological form has long been regarded as significant in pathogenesis, particularly in relation to the penetration of host tissues (Baillie, *et al.*, 1999). Besides, morphogenetic conversion, adhesive interactions and *quorum sensing* plays an important role in the development of *C.albicans* biofilms and therefore on tissue invasion property (Ramage, *et al.*, 2005; Uppulluri, *et al.*, 2009).

The ECM contributes to the architectural preservation of biofilms by the maintenance of stable cell–cell and cell–surface interactions and acts as a protective barrier (Martins, *et al.*, 2010), helping to reduce the permeability and preventing its penetration of antimicrobial agents and preventing its dissection (Palkovà, *et al.*, 2004; Beauvais, *et al.*, 2009). Still, Al-Fattani and collaborators found that the matrix did not constitute a barrier for some clinically used antifungals of different chemical structure, suggest that matrix play a minor role in drug resistance (Al-Fattani, *et al.*, 2006). The invasion of *C. albicans* depends as well of host immune mechanisms which become impaired (Calderone, *et al.*, 1997), besides the ability to adhere and invade tissues. Ferreira and collaborators investigated the ability of adhesion to polystyrene and invasion of agar on *Cagup1*Δ null mutant strain and showed that this strain was not able to penetrate the agar. These authors, also showed that in *Cagup1*Δ

null mutant strain the biofilm formation was affected: (i) the mutant had less total biomass compared with Wt, (ii) the *Cagup1*Δ null mutant strain was not able o form typical biofilm structures and (iii) *Cagup1*Δ had much less *hyphae*/pseudo-*hyphae* cell than Wt cells (Fig. 7) (Ferreira, *et al.*, 2010).

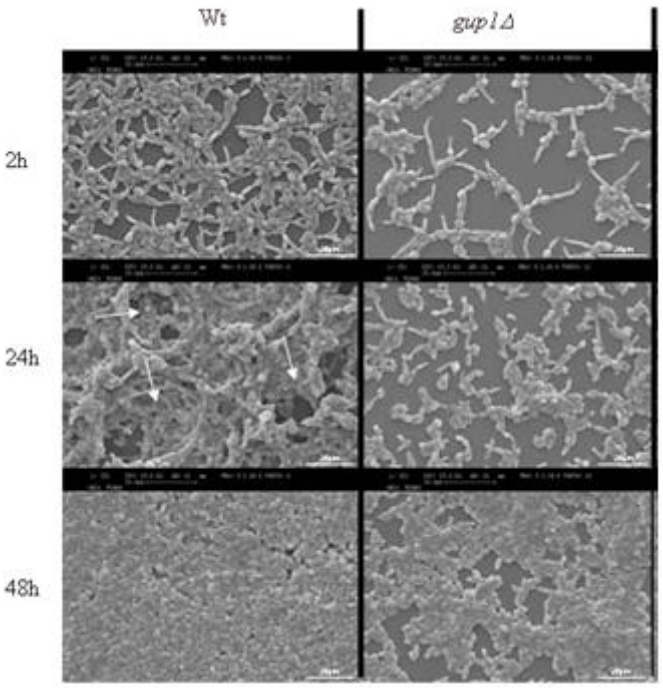

Figure 7 *Cagup1Δ* null mutation causes less and differently structured time-course biofilm formation.SEM micrographs of time course biofilm formation. Arrows indicate the channels observed in a typical biofilm structure - wt - not observed in *Cagup1Δ* null mutant strain biofilm (Ferreira, *et al.*, 2010).

3. Mammalian *GUP1*

Mammalian *GUP1* was found to act as a negative regulator of N-terminal palmitoylation of Sonic hedgehog (Shh) pathway. The Gup1p in mammals is described as being a homologue of *Saccharomyces cerevisiae* Gup1p. This protein is predicted as a member of the family of *O*-acyltransferases and shows high homology to the hedgehog acyltransferase in mammals (Hofmann, 2000, Abe *et al.*, 2008).

Since their isolation in 1990's, members of the Hedgehog family of intercellular signalling have been recognized as key mediators of many fundamental processes in embryonic development. The activity of intercellular proteins of Hedgehog (Hh) family has been implicated in various crucial processes, such as vertebrates development, control of sperm cells proliferation in adult tissues (Varjosalo and Taipale, 2007), follicular development, sclerotome specification myotome, cartilage differentiation and neuronal differentiation (Ingham, 1998). These proteins are also responsible for the formation of organs in vertebrates and insects (Ingham and McMahon, 2001). A defective activation of this signalling pathway can cause various types of diseases, including cancer (Varjosalo and Taipale, 2007).

Abe and colleagues cloned the *GUP1* gene from mouse embryos in insect cells and examined whether it was involved in regulating the palmitoylation at the N-terminal Sonic hedgehog (Shh). The palmitoylation is related to the proteins function contributing to subcellular traffic for the efficient excretion and signalling, increasing the hydrophobicity and for the association of proteins to membranes (Nusse, 2003). The palmitoylation is the responsibility of a protein called hedgehog acyltransferase (HHAT) (Abe, *et al.*, 2008), Hh protein is produced initially as a precursor molecule that consists of an N-terminal signal domain and a C-terminal protease domain, and these domains undergo by a series of changes throughout its synthesis. The N-terminus of Hh becomes modified by the fatty acid palmitate, on a conserved cysteine residue that is exposed at the very N-terminal end of the protein, after its signal sequence has been removed. The C-terminal protease of Hh cleaves the precursor in an autocatalytic manner to release the Hh signalling domain (HhNp), during this process the C-terminus HhNp is added covalently to a cholesterol molecule (Fig. 8) (Nusse, 2003, Porter, *et al.*, 1996). The palmitoylation is important for the regulation of the movement of HhNp in embryonic development. A loss of this signalling

mechanism may lead to the inability to form the multimeric protein complex of hedgehog protein (Abe, *et al.*, 2008).

Abe and co-authors concluded that the protein Gup1p, located in the endoplasmic reticulum, interacts with Shh protein and affects the palmitoylation of ShhNp, which was consistent with the fact that Shh and Gup1p co-expression results in a decrease of ShhN (Abe, *et al.*, 2008).

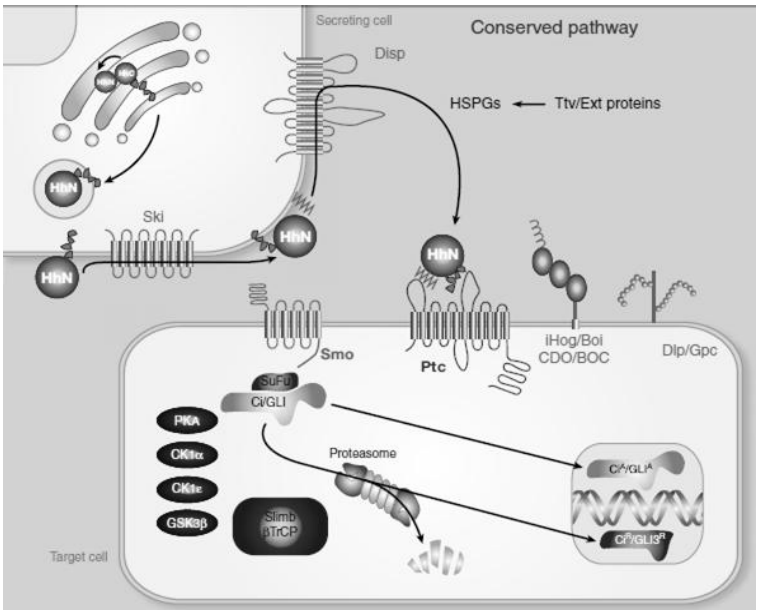

Figure 8 Hedgehog proteins undergo various processing steps. Initially the N-terminal signal sequence is cleaved. Then, the C-terminal domain of Hh catalyzes a reaction when cholesterol is covalentely added to a C-terminal (Verjosalo and Taipale, 2007).

Considering that, our group decided to assess the identification of *GUP1* molecular partners on a putative morphogenic signalling pathway in yeast, similar to Hedgehog, the present Master project was design to accomplished one of the first tasks of that investigation. Thus, the main aim of the present work concerned the creation of a small set of *E. coli* and *S cerevisiae* strains harbouring the *GUP1* fused to a reporter gene, or to a tag, namely GFP and Histidine tag. These strains would then be used to obtain a large amount of Gup1 protein and eventually identify the Gup1p molecular partners by Affinity Chromatography.

Material and Methods

1. Strains and growth conditions

In this study the *Saccharomyces cerevisiae* strains used were: W303-1A (*MAT***a** *ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 rad5-G535R*) (Thomas and Rothstein, 1989) and BHY54 (isogenic to W303-1A but *gup1::His5*$^{+}$) called as *Δgup1* mutant (Holst, *et al.*, 2000), and the *Escherichia coli* strains were: *XL1 Blue* (*end*A1 *gyr*A96 (nalR) *thi*-1 *rec*A1 *lac gln*V44 F'[::*Tn*10 *pro*AB+ *lac*Iq Δ(*lac*Z)M15] *hsd*R17(rK- mK+) and BL21(DE3)-CodonPlus-RIL (B F^{-} *ompT hsdS*(r_B^{-} m_B^{-}) *dcm*$^{+}$Tet^{r} *gal endA* Hte [*argU ileY leuW* Cam^{r}]). The later, is a strain for T7 based protein expression, constructed for high-level expression of recombinant proteins, which offers some advantages of simplicity, and improved codon bias difficulties. This, as *E.coli*BL21(DE3)-CodonPlus-RIL contains extra copies of the *argU*, *ileY* and *leuW* tRNA genes, which encode tRNA species that recognize arginine codons (AGA and AGC), the isoleucine codon (AUA), and the leucine codon (CUA) (Carstens and and Waesche, 1999). Most expression problems of eukaryotic proteins in *E.coli* strains result of the differences between the codon usage of the *E.coli* and the overexpressed protein. These differences in the codon usage can impede the translation due to demand for one or more tRNA that may be rare or lacking in the expression host (Kane, 1995, Goldman *et al.*, 1995). Insufficient tRNA can lead to translational stalling, premature translation termination, translation frameshift, and aminoacid misincorporation (Kurland and Gallant, 1996).

Yeast strains were batch-grown at 30°C, aerobically, on rich medium (YPD: yeast extract (1% w/v), peptone (2% w/v), glucose (2% w/v)) or minimal medium (YNBD: 0.67% yeast nitrogen base (w/v), 0.5% NaCl (w/v) and 2% glucose (w/v)), supplemented with aminoacids according with the auxotrophic markers. Incubation was performed at 200 rpm. Solid cultures media were supplemented with 2% agar and incubated at 30°C. Transformants were selected on YNBD supplemented with the according auxotrophic markers at 30°C, until colonies were observed.

Bacterial strains were batch-grown at 37ºC, aerobically, in Luria-Bertani (LB) medium (LB: 0.5% yeast extract (w/v); 1% tryptone (w/v); 1% NaCl (w/v)). Incubation was done at 200 rpm. Solid cultures media were supplemented with 2% agar and incubated at 37ºC. Transformants were selected on LB with the ampicilin (100mg/mL) or kanamycin (50µg/mL), until colonies were observed.

2. DNA Manipulation

A pair of plasmids, pET-25b(+) and pET-29b(+), were used to clone *GUP1* gene in *E. coli* BL21-CodonPlusRIL. Both plasmids belong to the pET System, which is the most powerful system yet developed for the cloning and expression of recombinant proteins in *E. coli*. These vectors have an N-terminal (His)10-tag, which can be used as affinity ligands for purification purposes (Fig. 9).

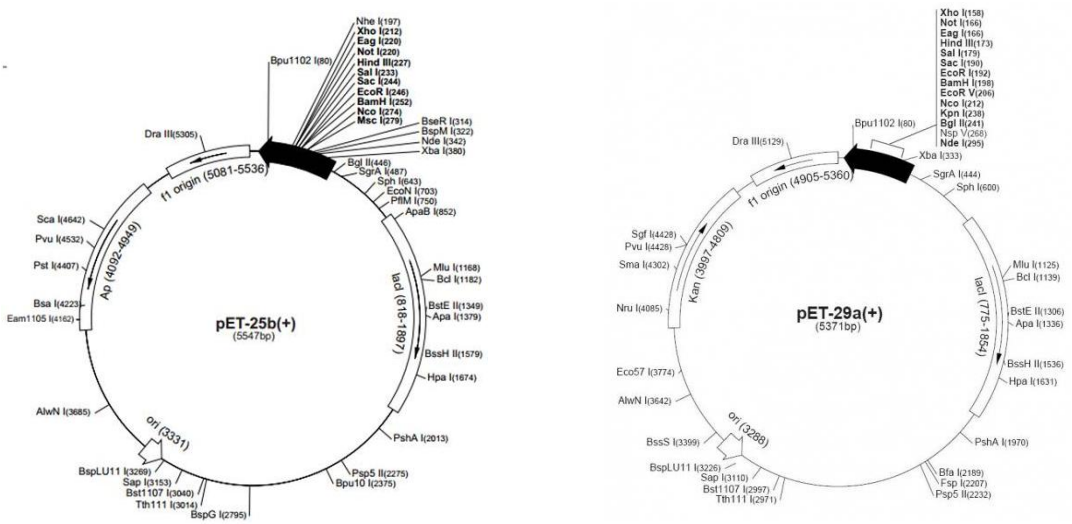

Figure 9 Schematic representation of pYES2 and pYES3/CT plasmids.

Another set of plasmids, pYES2 and pYES3/CT (Fig.10), were used in order to clone *GUP1* gene in *S.cerevisiae* strain. The pYES2 and pYES3/CT vectors were designed for inducible expression of recombinant proteins in *S.cerevisiae*. Features of the pYES2 vector allow easy cloning of a gene and selection of transformants by uracil prototrophy. On the other hand, the pYES3/CT allows purification and detection of the expressed proteins as it contains the C-terminal peptide encoding the V5 epitope and a polyhistidine (6xHis) tag. Both expression plasmids contain yeast GAL1 promoter for high level inducible protein expression by galactose and repression by glucose (Giniger, *et al.*, 1985; West, *et al.*, 1984) and URA3 or TRP1 auxotrophic marker, for selection of yeast transformants, for pYES2 and pYES3/CT, respectively.

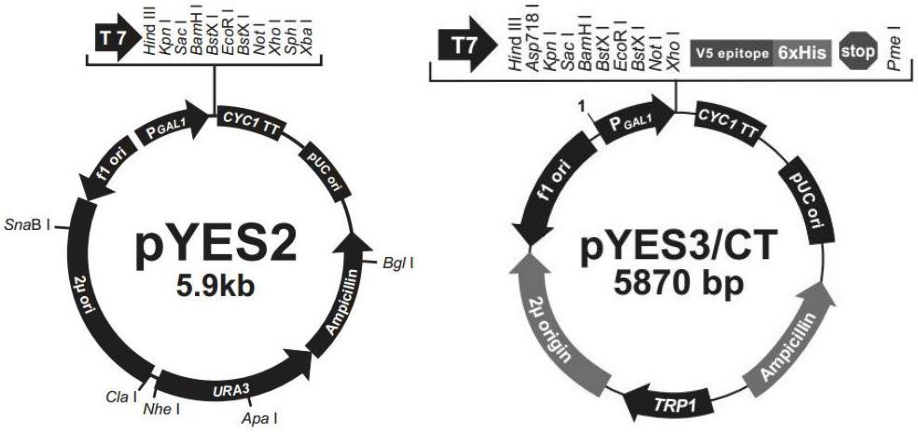

Figure 10 Schematic representation of pYES2 and pYES3/CT plasmids.

Additionally, a plasmid containing *GUP1*-GFP chimera was also used. This plasmid, pYES2-*GUP1*-GFP, was kindly supplied by our Italian collaborator's Francesco Grieco and Gianluca Bleve from Instituto di Scienze Delle Produzioni Alimentari del Consiglio Nazionale Delle Ricerche (ISPA), Leece, Italy (Bleve, *et al.*, 2005).

3. Strategies for expression of *GUP1* in *S.cerevisiae* with His-tag

The referred vectors, pYES2 and pYES3/CT were used to clone *ScGUP1* in *S.cerevisiae* null mutant strain, using a tag of histidines, in order to obtain the purified protein and eventually identify some molecular partners.

3.1. Expression using pYES3 plasmid.

3.1.1. *GUP1* gene PCR amplification

ScGUP1 gene was amplified from genomic DNA of W303-1A (previously isolated and already available in the group) by PCR, using the primers listed on Table 1. The forward primer includes the *HindIII* restriction site, whereas the reverse primer contains the *XhoI* restriction site. The PCR programme followed is described below:

For a volume of 20µl, the reaction mixture included the following:

- 1µl of Taq polymerase
- 10µl of Buffer,
- 4 µl of $MgCl_2$,
- 2µl of dNTP's

- 4µl of primers,
- 70µl H2O ultrapure autoclaved water.

The PCR reaction followed the next steps: denaturation (94 °C, 5 minutes), annealing (30 cycles: 94 °C, 1 minute; 60 °C, 3 minutes; 72 °C, 3 minutes) and elongation (72 °C, 10 minutes).

Forward *HindIII*	5' AAATTTAAGCTTATGTCGCTGATCAGCATCCTG 3'
Reverse *XhoI*	5' AAATTTCTCGAGGCATTTTAGGTAAATTCCGTG 3'

Table 1 Primer pair for amplification of *GUP1*gene.

3.1.2. Purification of DNA fragments

The product of PCR was visualized and checked for correct size by DNA electrophoresis in a 1% agarose gel. The electrophoresis ran for approximately 60 min at 65 volts. The DNA was visualized by UV illumination (245nm) at *Eagle Eye II* [*Stratagene*], previously stained with Gel RED [Biotium] dye, using Lambda DNA/Eco471 [Fermentas] as molecular weight marker

The DNA fragments were extracted and purified from the gel with *DNA Clean & Concentrator™ - 5 kit*, [ZymoResearch] system, following the manufacture recommendations.

Alternatively, the DNA fragments were purified directly from the PCR product by precipitation, following the next protocol:

- Add 1/10 volume of Sodium Acetate (3 M, pH 5.2).
- Add 2.5–3.0 times the volume of 95% ethanol.
- Incubate for 2 hours, at least at -20ºC (incubation can be done O.N.)
- Centrifuge at > 14,000 x g for 30 minutes, at room temperature.
- Discard the supernatant, taking care to do not throw out DNA pellet, which may not be visible.
- Rinse with 70% Ethanol
- Spin a maximum speed for 15 min.

- Discard the supernatant and dissolve the pellet in TE buffer or water. Make sure the buffer (or water) comes into contact with the whole surface of the tube, since a significant portion of DNA may be deposited on the walls.

3.1.3. Enzymatic digestion of DNA

To clone *GUP1* gene into different plasmids, both the DNA fragment and the plasmids were digested with appropriated restriction enzymes (*HindIII* and *XhoI*). These enzymes were chosen as they belong to the multi cloning site (MCS) of the plasmids in use and, at the same time, do not cut the insertion fragment.

Plasmids linearization was obtained by the following reaction mixture:

- 8 µl of vector,
- X µl of enzyme *HindIII*,
- Y µl of enzyme *XhoI*,
- 2 µl of buffer,
- Z µl ultrapure autoclaved water,

The enzymatic digestion of insertion fragment was obtained following the reaction mixture:

- 2 µl of insertion fragment (400ng/µl),
- X µl of enzyme *HindIII*,
- Y µl of enzyme *XhoI*,
- 4 µl of buffer,
- Z µl ultrapure autoclaved water.

The volume ratio used was calculated using the *Fermentas double digestion* tool (http://www.thermoscientificbio.com/webtools/doubledigest/?redirect=true).
Ultrapure water was added to a final reaction volume of 20µl. The mix was incubated for 3 hours at 37°C.

3.1.4. Ligation reaction

Digested plasmids were treated with *Shrimp Alkaline Phosphatase* (SAP), before the ligation reaction, in order to remove the phosphate 5' end. This elimination prevents the auto-ligation of the vector, reducing the frequency of plasmid circularization.

Therefore, plasmid was incubated with the SAP enzyme for 10 minutes, at 37°C, and subsequently incubated for 15 min at 65°C, in order to inactivate the SAP enzyme completely.

The ligation, below described, was next performed by incubating the treated plasmid and the purified gene overnight at 4°C.

- 1 µl ligation buffer,
- 1 µl plasmid,
- 7 µl of purified digestion product,
- 1µl of T4 DNA ligase,
- 10 µl of ultrapure water to achieve a final volume of 20 µl.

3.1.5. Propagation in *E.coli* XL1 Blue

For DNA propagation, the ligation product was used to transform *E.coli XL1 Blue* competent cells. The *E. coli XL1 Blue* competent cells preparation was performed according with the procedure of Inoue and collaborators, which consists on inducing membrane alterations in order that cells become more susceptible to accept foreign macromolecules (Inoue *et al.*, 1990).

E.coli XL1 Blue transformation was made by standard protocols (Ausubel *et al.*, 1996) as follows:

A volume of 5 µl plasmid DNA (≈50 µg/µl) was added to 200 µl of *E. coli XL1 Blue* competent cells. These cells were incubated on ice for 40 minutes, subjected to a heat shock, by incubation at 42°C for 2 minutes, and then maintained on ice for another 2 minutes. In order to allow cells to recover, 500 µl of LB medium was added and the cell suspension was further incubated at 37 °C, for 1 hour. A volume of 200 µl of this solution was platted on solid LB medium supplemented with 100 mg/mL of ampicilin. Cells were allowed to grow overnight at 37ºC.

3.1.6. Plasmid isolation from *E.coliXL1 Blue*

In order to obtain a large scale of plasmid DNA, several colonies of *E.coli XL1 Blue* transformed with *GUP1* were inoculated in 5 ml of LB liquid medium supplemented with ampicilin/kanamycin and grown overnight at 37 ºC. The plasmids were isolated

with *GeneElute™ Plasmid Miniprep Kit* [Sigma] following the protocol of the manufacturer, and the expected sizes were verified by gel electrophoresis after digestion with the restriction enzymes.

3.1.7. Transformation of *S.cerevisiae Δgup1* mutant strain

S.cerevisiae Δgup1 mutant strain was transformed with the expression vector pYES3/CT harbouring *GUP1*obtained as described above. Transformation was performed according to Gietz and co-workers (Gietz, *et al*., 1992). The procedure is next described:

S.cerevisiae Δgup1 mutant strain cells were grown overnight at 30ºC on liquid YPD medium supplemented with glucose 2%, as carbon source.

- Collect of the cells by centrifugation, at 3000 rpm during 5 min,
- Ressuspend the pellet in 1ml of ultrapure sterilized water and transfer it into an e*ppendorf*,
- Centrifuge again for 2 min, at 8000rpm,
- Wash the pellet with sterilized water,
- Centrifuge once more for 2 min, at 8000 rpm,
- Wash the pellet with LiAc (0.1M)/TE (1x) buffer,
- Perform another centrifugation in the same conditions,
- Ressuspend the pellet in 200 µl LiAc (0.1M)/TE (1x); Incubate on ice for 15 minutes,
- To 100 µl of the mixture was added 10 µl of salmon sperm DNA as carrier (10 mg/ml) and 0,1-0,2 µg/µl of the plasmid. A control without the plasmid was done,
- Add 600µL LiAc (0.1M)/TE(1x)-PEG 50%,
- Incubate for 30 min, at 28ºC, with gentle agitation,
- Heat shock the cells by incubating at 42ºC during 15 min,
- Incubate for 10 min, at 4ºC,
- Centrifuge for 2 min, at 8000 rpm,
- Ressuspend the pellet in sterile water (200µL),
- Plate the cells into selective medium plates (YNB) and incubate at 30ºC until colonies were observed.

3.1.8. Colony PCR

In order to confirm positive clones, a colony PCR was performed, using the same primers utilized to amplify the *GUP1* gene, listed on Table 1, following the protocol by Lõoke and co-workers (Lõoke, *et al.*, 2011), next described:

- Pick the yeast colony from plate or spin 100-200 µl of liquid yeast culture (OD_{640nm} 0.4) and suspend in 100 µl of 200 mM LiAc, 1%SDS,
- Incubate for 5 minutes, at 70°C,
- Add 300 µl of 96-100% ethanol and vortex shortly,
- Centrifuge at 15000*g*, for 3 minutes,
- Wash the pellet with 70% ethanol,
- Dissolve the pellet in 100 µl H_2O or TE buffer and spin down cell debris for 15 seconds at 15000 *g*.

3.2. Expression using pYES2 plasmid.

The strategy followed to express *ScGUP1* gene using pYES2 vector was exactly the same as for pYES3 plasmid (described above) with the only exception of the reverse primer used to amplify *ScGUP1* gene, which includes a tag of 6 histidines (Table 2).

Forward *HindIII*	5' AAATTT AAGCTTATGTCGCTGATC AGCATCCTG 3'
Reverse *XhoI*	5' TATCTCGAGTCACATCATCACCATCACCATGCATTTTAGGTAAATTCCGTG 3'

Table 2 Primer pair for amplification of *GUP1*gene. Reverse primer includes a tag for six histidines.

4. Strategies for expression of *GUP1* in *E. coli* BL21(DE3)-CodonPlus-RIL strain

Again, *ScGUP1* was amplified by PCR with the primers listed in Table 1 and cloned into two different expression plasmids, pET25b(+) and pET29b(+) using the same procedure described in section 3.1, once the fragment could be cloned into this plasmid MCS with the same enzymes. The obtained constructions were transformed in *E.coli*BL21(DE3) competent cells, using the same protocol described on section 3. In the same way, also the positive clones were verified by colony PCR as described.

5. Study of *GUP1* expression

5.1. Expression of *GUP1*-His-tag construction in *E. coli* BL21(DE3)-CodonPlusRIL strain

To induce the protein expression, two different media were tested, LB with glucose 2% as carbon source and LB with lactose 2% as carbon source. Cells harbouring the pET25b(+)-*GUP1*, the pET29b(+)-*GUP1* and the correspondent empty plasmids were grown in both media, overnight, at 37°C. To a volume of 15 ml of each culture, OD_{600} ≈0.6, it was added different concentrations of β-D-1-thiogalactopyranoside (IPTG) (0.1mM, 0.5mM, 1mM and 2 mM) to induce the expression of *GUP1*-His-tag chimera. Three temperatures 18, 30 and 37 °C were tested. Samples were treated with 5% SDS-page buffer with and without β-mercaptoethanol and further analyzed by SDS-PAGE and also Western blot.

5.2. Expression of *GUP1*-His-tag plasmid construction in *S.cerevisiae Δgup1*

For expression in *S.cerevisiae Δgup1* mutant strain, the transformants harbouring the construction on pYES3/CT and pYES2 vectors, were grown overnight in minimal medium under repression conditions (with glucose as carbon source). Induction was achieved by incubating the cells for 2, 4, 6 and 8 h in minimal medium supplemented with 2% galactose, as carbon source. Samples were treated by precipitation with TCA and further analysed by Western blot.

5.3. Expression of Gup1p-GFP fusion in *S.cerevisiae*

Yeast strains harbouring pYES2-*GUP1*-GFP (Bleve, *et al.,* 2005) were obtained by transformation of *S.cerevisiae Δgup1* mutant strain as described above. These were batch-grown at 30°C, aerobically in minimal medium with glucose 2% as carbon source. Cell were harvested and incubated in fresh inductive media (galactose 2%) for 2, 4, 6 and 8 h. Samples were analyzed by Fluorescent microscopy and precipitated with TCA as mentioned above for Western blot.

6. Western blot

Western blot samples were separated by SDS-PAGE (10%) and then transferred to PVDF membrane. The primary antibodies used were (i) anti-Histidine (1:3000) (anti-His HRP [5Prime]) (ii) anti-GFP (1:5000) [Roche] and monoclonal anti mouse antibody (1:10000) [Sigma] as secondary antibody. The reacting polypeptides were visualized using ECL plus Western Blotting Detection system [Amersham Biosciences] on a Chemidoc software [BioRad].

7. Fluorescence Microscopy

The expression and the localization of Gup1-GFP were verified in *S.cerevisiae* Δ*gup1* strain harbouring pYES2-*GUP1*-GFP (*ScGUP1*-GFP) by Fluorescence Microscopy. Cells were grown overnight in YNB-glucose medium until an absorbance OD_{640nm} of approximately 0.4, then cells were washed twice with sterile distilled water and transferred to 15ml of YNB medium containing 2% galactose as the unique carbon and energy source. Samples were taken over 8 hours and visualized in a Leica Microsystems DM-5000B epifluorescence microscope with appropriate filter settings using a 100x oil-immersion objective. Images were acquired with a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software.

8. Immunoprecipitation

Yeast *ScGUP1*-GFP cells were grown overnight in glucose as carbon source. Cells were then washed twice, diluted to an OD_{640nm} 0.4 in galactose-based medium and incubated during 4 hours, at 30ºC for induction. Over this period cells were subjected to immunoprecipitation using *DynaBeads® Magnetic Beads* [Merck Millipore], according with manufacturer indications. Briefly:

- Collect yeast culture at an OD_{640nm} of 1 and centrifuge for 5 minutes at 4000 *g*, and at 4ºC,
- Ressuspend the pellet in immunoprecipitation (IP) buffer 1X with protease inhibitors in order to lysate the cells,
- Transfer the suspension for glass tubes and add half cell volume of glass beads, vortex five times, during one minute. Place the tube on ice for one minute between each vortex,
- Remove the yeast cell lysate from the beads and transfer into a fresh tube,

- Centrifuge the lysate for 10 minutes, at 100 *g*, and transfer the supernatant into fresh tube of 2 ml,
- Add 50µl of IP buffer 10X containing the detergent NP-40 [Roche] and incubate for1h, at 4°C,
- Add 5µl of antibody (Anti-GFP) and incubate overnight at 4°C,
- Add 100µl of DynaBeads (Protein G-Sepharose) [Merck Millipore] and incubate 6h at 4°C,
- Wash the cells 6 times for 5 minutes (shaking) with IP buffer and 1 time for 2 minutes with PBS buffer to remove detergent,
- Add 50µL of sample buffer 1X and denature at 95°C, during 5 minutes.

The protein amounts were analyzed by 10% SDS Page followed by Coomassie Blue and silver staining described next:

- Place a polyacrylamide gel in a glass or polyethylene container and immerse gel in methanol/acetic acid distaining solution for 20 min with gentle agitation,
- Pour out the distaining solution and cover the gel with 50% methanol. Incubate for 10 minutes, with gentle agitation,
- Pour out the methanol. Wash the gel for 10 minutes with H_2O,
- Submerge the gel in 0.02% sodium tiossulfate solution during 1 minute,
- Wash the gel twice with H_2O,
- Add 0.1% silver nitrate and incubate for 20 minutes, at 4°C,
- Pour out silver nitrate. Cover gel with 3% sodium carbonate and 50µl formaldehyde and shake until obtain the ideal revelation.

Simultaneously, one gel was transferred for PVDF membranes and subjected to Western blot analysis.

Results and Discussion

S.cerevisiae GUP1 is involved in a wide range of cellular processes (Oelkers, *et al.*, 2000; Bonangelino, *et al.*, 2002; Askree, *et al.*, 2004; Bosson, *et al.*, 2006; Ferreira *et al.*, 2006; Jaqueneoud, *et al.*, 2008; Ferreira and Lucas, 2008; Tulha, *et al.*, 2012). Lately, this gene has attracted a great deal of attention because its mouse homologue acts as a negative regulator of the N-palmitoylation of the Sonic hedgehog pathway (Abe, *et al.*, 2008). Sonic hedgehog pathway plays an important role in the development of diverse animal phyla, regulating morphogenesis, differentiation and patterning during embryogenesis, including proliferation and cell fate (Verjosalo and Taipale, 2007). This finding contributed to emphasize the role of Gup1p in yeast cellular morphology and division control, but more importantly is in agreement with the hypothesis of the existence of an Hh-like pathway in yeasts. Therefore, our group started to express Gup1 protein in two different models, *S. cerevisiae* and *E. coli* BL21(DE3)-CodonPlus-RIL, to subsequently be able to purify Gup1 protein and attached partners. This, in order to identify those molecular partners that putatively would interact with *GUP1* on this novel morphogenic pathway in yeasts.

1. Methodologies used on protein expression studies

In *S.cerevisiae* there are several methodologies available for protein expression that allows not only the immunodetection but also the immunopurification. Over the time, a high number of studies were performed by our group, as well as other groups to assess the expression of Gup1 protein, using different methods and expression systems. This includes for example, the attempt to produce antibodies directed against putative cytosolic and extracellular domains or against synthetic peptides of Gup1 protein, but without success (Ferreira, C., Phd Thesis 2005). The use of reporter proteins is commonly used to indirectly study the expression of a protein, like *e.g. green fluorescent protein* (GFP), β-galactosidase (encoded by the bacterial gene *lacZ*) and luciferase (from lightening bugs). Since reporter proteins can vary regarding their relative stability, this feature has to be taken into consideration when selecting an inducible reporter construct. Finally, the expression of the protein being tested is determined indirectly, either by the reporter protein *in vitro* activity (*e.g.* β-galactosidase and luciferase), by immunological assays, or *in vivo* histochemical procedures (*e.g.* GFP) (Wahlfors, *et al.*, 2001).

When the aim of the study includes the purification of the protein, the use of epitope-tags is the chosen strategy, being the tag of Histidines (HIS-tag) or of Hemaglutinines (HA-tag) the most commonly used. Basically, a protein can be engineered to include a short stretch of residues corresponding to an epitope that facilitates its subsequent biochemical and immunological analysis. This may be appended at the N or C-terminus of the coding region of the protein of interest. The recombinant proteins generated can be easily identified and purified by immunoprecipitation, using antibodies against the epitope (Sells and Chernorff, 1995, Chubet and Brizzard, 1996), frequently available commercially. Some specific examples of biological applications for epitope tagging methodology include: (i) determining the size, the intracellular localization, and the protein abundance (Molloy *et al.,* 1994; Canfield *et al.,* 1996); (ii) monitoring post-translational modifications; (iii) analyzing the function of individual protein domains; (iv) studying receptor binding and internalization of exogenous proteins (Brown et *al.,* 1995); and (v) identity a specific protein within a complex of proteins (Zhou *et al.,* 1992). In this work we analyzed *S.cerevisiae GUP1* expression using an HIS-tag and the GFP reporter protein.

2. Strains harbouring *Saccharomyces cerevisiae GUP1*

The expression of *GUP1* was assessed using a set of different plasmids: pYES2 and pYES3/CT for expression in *S. cerevisiae ⊿gup1*null mutant strain and pET25b and pET29b for expression in *E.coli* BL21(DE3)-CodonPlus-RIL. The pYES2 and pYES3/CT vectors contain yeast *GAL1* promoter for high level of inducible protein expression by galactose and repression by glucose (Giniger *et al.,* 1985; West *et al.,* 1984). These plasmids have the *URA3* and the *TRP1* auxotrophic marker for pYES2 and pYES3/CT, respectively. The pYES3/CT allows purification and detection of the expressed proteins because it contains a C-terminal peptide encoding the V5 epitope and a polyhistidine (6xHis) tag. As above mentioned, for the expression in *E.coli* BL21(DE3) the pET25b and pET29b plasmids were used. Target genes cloned in pET plasmids are under the control of strong bacteriophage T7 transcription and translation signals, being the expression induced by isopropyl β-D-1-thiogalactopyranoside (IPTG).

In general, the strategy applied to express the *ScGUP1* gene consisted in cloning constructions of *ScGUP1* gene tagged with either the HIS tag or GFP reporter protein, both on the *S.cerevisiae Δgup1*strain and on *E.coli* BL21(DE3) strain. A restriction enzyme map for the genomic DNA coding sequence of *GUP1* was made, in order to select the appropriated enzymes for cloning steps. These, must do not cut the fragment and at the same time be included into the plasmid Multi-Cloning-Site (MCS). Selected the enzymes, their sequence was included in the primers designed to amplify the *GUP1* gene by PCR. Each construction, pYES and pET harbouring *ScGUP1* gene, was then transformed in *E.coli XL1 Blue* to obtain a large scale of DNA. The *E.coli XL1 Blue* transformants were selected in LB medium supplemented with 100 µg/ml of ampicilin for pYES2, pYES3/CT and pET25b constructions, and 50 mg/ml of kanamycin for pET29b. The positive clones, carrying the *GUP1* gene fragment, were verified through electrophoresis of the enzymatic digestion, confirming the bands size.

2.1. Construction of expression vectors

2.1.1. Construction of pET plasmids

The *ScGUP1* gene was PCR-amplified with an appropriate primer pair (Table 1, Material & Methods). Selected the enzymes, *XhoI* and *HindIII,* their sequence was included in the primers designed to amplify the *GUP1* gene by PCR. The product of PCR reaction was visualized by electrophoretic gel analysis, being obtained a band with approximately the size of *ScGUP1* ≈1683 bp (Fig. 11).This fragment was purified from the agarose gel.

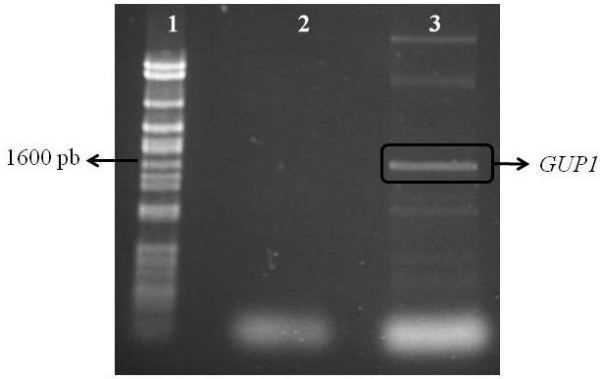

Figure 11 Electrophoretic analysis of PCR-amplified *GUP1*. Molecular weight marker Lambda DNA/Eco471, Marker 13 [Fermentas] – column 1; Negative control – column 2; Product of PCR-amplified *GUP1* – column 3.

Both, the PCR purified fragment and the plasmids (pET25b and pET29b) were double digested with *XhoI* and *HindIII* and then ligated with T4 DNA enzyme. This way, were obtained two constructions: pET25b harbouring *GUP1* and pET29b harbouring *GUP1*. Both constructions were used to transform *E.coli XL1 Blue* strain. We optioned to transform first *XL1 Blue E.coli* strain and only afterwards the expression *E. coli* strain BL21(DE3)-CodonPlus-RIL, as the former has typically a higher cloning efficiency and lacks endonucleases.

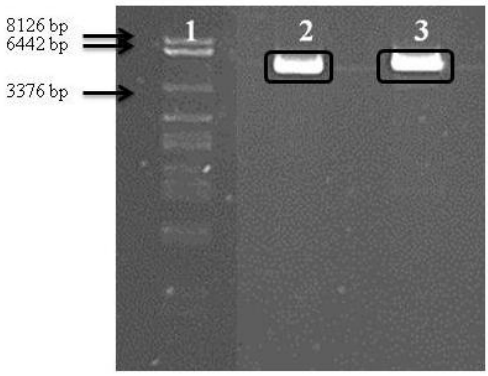

Figure 12 Electrophoresis analysis of pET25b and pET29b plasmids double digested with *HindIII* and *XhoI*. Molecular weight marker Lambda DNA/Eco471, Marker 13 [Fermentas] – column 1; digestion product of pET29b plasmid with the expected band at ≈ 5371 bp – column 2; and digestion product of pET25b plasmid with the expected band at ≈ 5500 – column 3.

The positive *Xl1 Blue* clones, for both constructions, were confirmed by restriction analysis. The plasmid was extracted by Mini-prep (see Materials & Methods) and then digested with *HindIII* and *XhoI* enzymes. The positive clones were therefore confirmed by the presence of the two expected bands on the electrophoretic gel: a band of ≈ 5500 bp corresponding to the plasmids size and a band of ≈ 1700 bp corresponding to the *GUP1* gene (1683 bp) (Fig. 13).

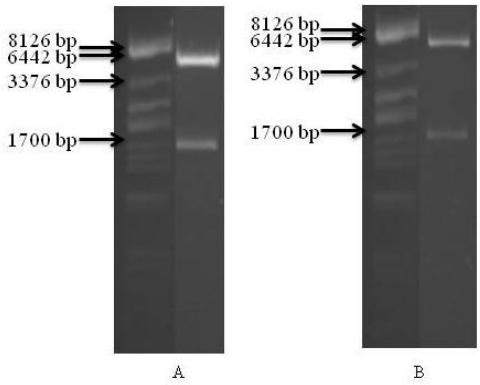

Figure 13 Product of double digestion with *HindIII* and *XhoI* of the clones harbouring pET25b(+)-*ScGUP1* construction – A; and p pET29a(+)-*ScGUP1* construction – B. Molecular weight marker Lambda DNA/Eco471, Marker 13 [Fermentas].

2.1.2. Construction of pEYS plasmids

The strategy followed to clone *ScGUP1* gene in the pET system vectors was applied to clone the same gene into the pYES vectors. Yet, to clone *ScGUP1* gene into pYES2 a different reverse primer had to be used, in order to include a tag of six histidines (Table 2, Material & Methods). In the same way, the PCR fragments and pYES2 and pYES3/CT plasmids were double digested with *XhoI* and *HindIII*, purified from the electrophoresis gel and subsequently ligated with T4 DNA enzyme. The products of the two ligations were then transformed in *E.coli XL1 Blue* strain.

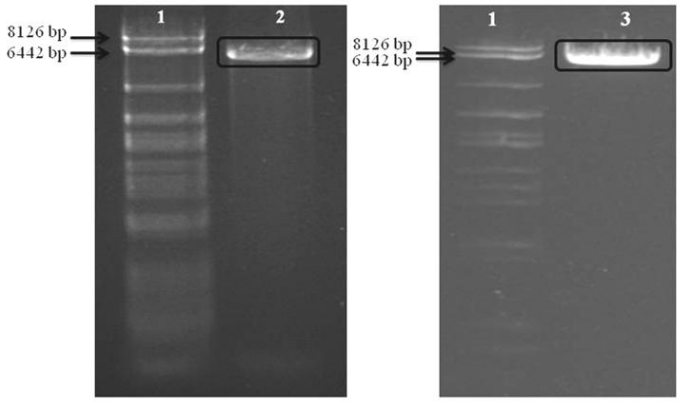

Figure 14 Product of double digestion with *HindIII* and *XhoI* of the pYES2 and pYES3/CT plasmids with ≈6000 bp – column 2 and 3 (5856 bp and 5870, respectively). Molecular weight marker Lambda DNA/Eco471, Marker 13 [Fermentas] – column 1.

The positive clones were confirmed through enzymatic digestion with *HindIII* and *XhoI* enzymes, by the presence of the two expected bands: a band ≈ 6000 bp

corresponding to the plasmids size and of a band ≈ 1700 bp corresponding to *GUP1* gene (1683 pb) (Fig. 15).

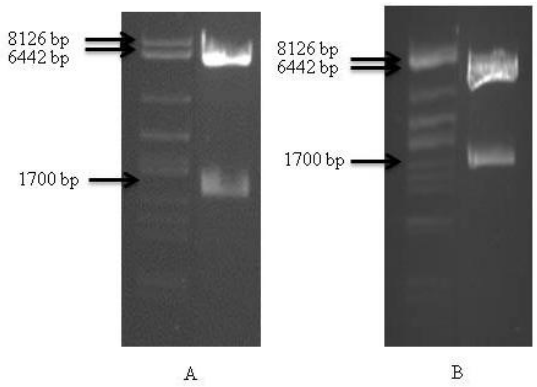

Figure 15 Product of double digestion with *HindIII* and *XhoI* of the positive clones harbouring pYES3/CT-*ScGUP1* construction – A; and pYES2-*ScGUP1* construction – B. Molecular weight marker Lambda DNA/Eco471, Marker 13 [Fermentas].

3. Expression studies of Gup1 protein

3.1. Expression in *Escherichia coli* BL21(DE3)-CodonPlus-RIL

Most of the expression plasmids for *E. coli* strains contain the presence of the *lac* repressor protein, which regulates the expression of the cloned protein. During normal cell growth, the *lac* repressor protein binds to the sequence of the operator in the plasmid, preventing the recombinant protein expression. In this study, for the expression of Gup1 protein in *E.coli* BL21 (DE3) cells cloned into pET25b and pET29b vectors, it was used isopropyl-β-D-thiogalactoside (IPTG). This compound binds the *lac* repressor protein inactivating it.

Despite of *E. coli* be one of the most successful vehicles for over-expression of both prokaryotic and eukaryotic proteins, sometimes the expression of heterologous proteins is difficult, or even impossible. One of the reasons arises from deficient codon usage. The Gup1p protein has a large number of residues of arginine, isoleucine, leucine and proline that could be very problematic for the expression in *E.coli*, as they are encoded by rare codons. To overcome this problem, we used as expression host the BL21-CodonPlus-RIL strain, which has been improved to express the eukaryotic tRNA genes for arginine, isoleucine and leucine rare codons.

The efficiency of an expression system is considered reasonable when an abundant biomass containing a large amount of the target protein is obtained. For protein production in *E.coli* variables such as temperature, pH, nutrient composition, among other parameters, can affect proteolytic activity, secretion and production levels (Bird, *et al.,* 2004). Therefore, the expression of the Gup1 protein cloned in *E. coli* BL21(DE3) host cells with the pET plasmids was investigated under several conditions (Table 3). Thus *E coli* transformants were grown in media with distinct carbon sources and at different temperatures. Induction was performed for crescent periods of time and concentrations of IPTG (Table 3).

Temperature	18°C and 37°C
Medium	Luria Bertani (LB) with glucose Luria Bertani (LB) with lactose
IPTG	0.1 mM, 0.5 mM, 1 mM and 2 mM
Time	2, 4, 6, 8 and 22 hours.

Table 3 Expression conditions performed of pET25b-*GUP1* and pET29b-*GUP1* in *E.coli* BL21(DE3).

The level of inducer required, in the present case IPTG, for optimal expression depends mainly on: (i) the strength of the promoter, (ii) the presence or absence of repressor genes on the plasmid, (iii) the cellular localization of the expressed protein, (iv) the response of the cell to the recombinant protein, and (v) its solubility. A wide range of IPTG concentrations, from 0.005 mM to 5 mM, have been used to induce protein expression (reviewed by Donovan, *et al.,* 1996). Thus in our study, it was important to understand the influence of IPTG concentration.

The *E.coli* BL21(DE3) cells, harbouring pET-25b-*GUP1* and pET-29b-*GUP1* constructions, were incubated at 37°C, in LB medium with 1mM of IPTG for 0, 2, 4, 6 and 22 hours. At these time points, samples were taken, harvested and treated with 5% SDS-Page buffer. Then, these samples were incubated at 95°C, during 5 minutes to promote the denaturation of the proteins, and analyzed by SDS-Page. It was expected to observe a band at ≈65 kDa corresponding to the Gup1 protein, yet despite of the induction time, this was not observed (Fig. 16 A and B). In fact, all the samples presented similar profile to the control, 0 h induction (without IPTG) (Fig. 16 A and

B- column 1). On the other hand, it was visible four over-expressed bands, of approximately 90kDa, 70kDa, 48 kDa and 35 kDa in both constructions (Fig. 16 A and B). The identification of these bands was not performed at this stage.

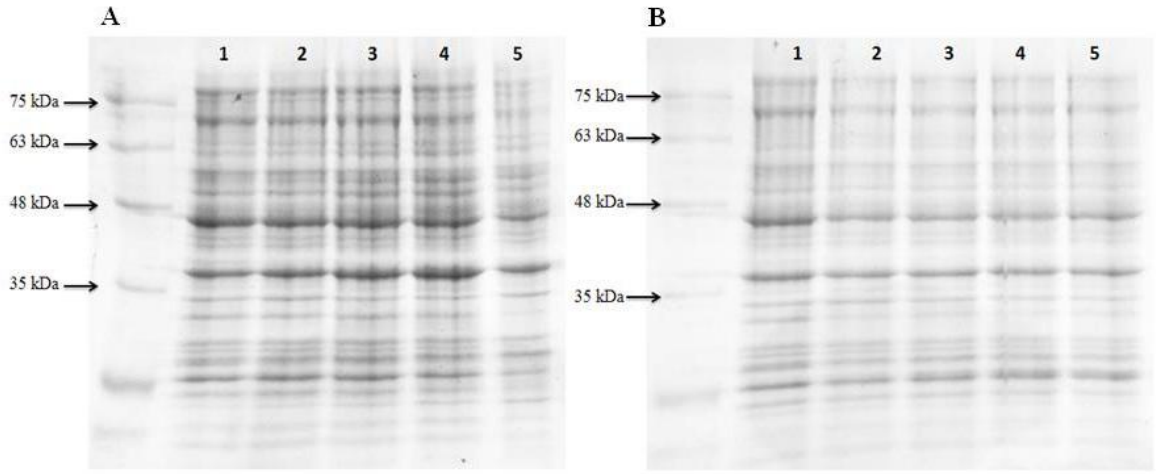

Figure 16 Gel SDS-Page of *E coli* cells grown on LB medium at 37°C with 1mM of IPTG on 0, 2, 4, 6 and 22 hours - column 1 to 5, respectively ; harbouring pET-25b-*GUP1*- A , or pET-29a-*GUP1* – B

In order to assess the influence of the concentration of IPTG on Gup1 protein expression, different concentrations of this inducer were checked, 0.1 mM, 0.5 mM and 2 mM, at 37°C in LB medium (Fig. 17 and 18). At this stage, other controls were added, namely *E.coli* Bl21(DE3) wild-type cells (untransformed) and *E.coli* Bl21(DE3) cells transformed with pET25b empty plasmid (Fig. 17 and 18). Once again the protein profiles of induced cells harbouring pET-25b-*GUP1* construction were equal to the controls, meaning that Gup1p over-expression was not attained (Fig. 17 and 18).

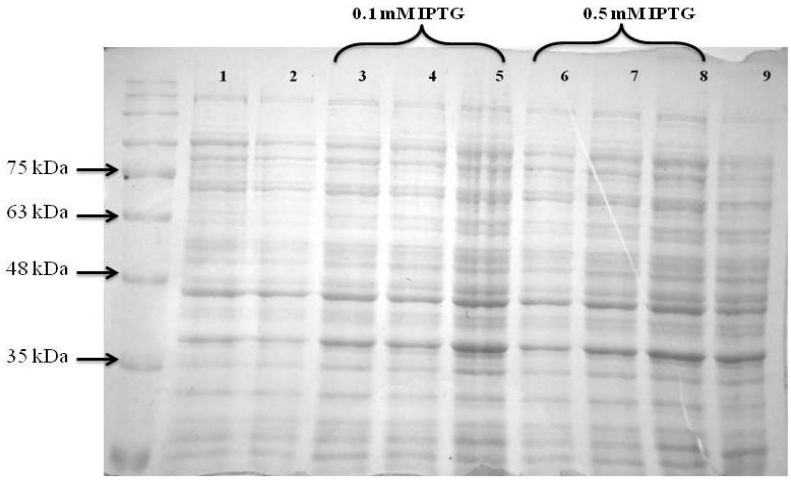

Figure 17 SDS-Page electrophoresis of *E.coli* BL21 (DE3) grown on LB medium at 37°C harbouring: plasmid pET25b - column 1; harbouring pET-25b-*GUP1* construction at 0 hours - column 2; harbouring pET-25b-*GUP1* construction, induced with 0.1 mM of IPTG for 2, 4 and 6 hours - column 3 to 5; and with 0.5mM of IPTG for 2, 4 and 6 - column 6 to 8, respectively; and *E.coli* BL21 (DE3) wild-type - column 9.

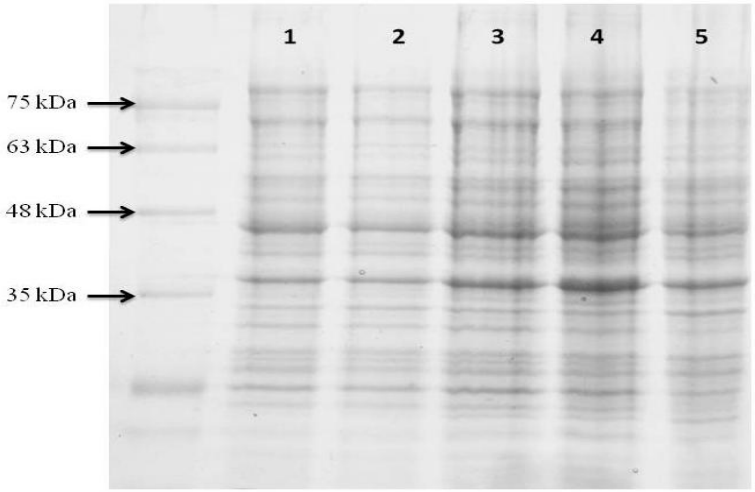

Figure 18 SDS-Page electrophoresis of *E.coli* BL21(DE3) harbouring pET-25b-*GUP1* construction, grown on LB medium, at 37°C, induced with 2mM of IPTG for 0, 2, 4, 6 and 22 hours - column 1 to 5, respectively.

Thought the Gup1p over-expression band, theoretically of ≈ 65 kDa, was not observed, and is not possible to be sure that the protein is not present, unless a western blot is performed. This because the protein could be in so low amounts that the sensitivity of Coomassie stain was not enough, or because the protein could migrate differently and therefore appear at a different relative position. Specially with plasma membrane proteins it has been widely described that proteins either migrate less or more on the SDS-Page gel than expected (Seddon, *et al.*, 2004; Wagner, *et al.*, 2007). Therefore, *E.coli* BL21(DE3) pET-25b-*GUP1* and *E.coli* BL21(DE3) pET-29b-

*GUP1*cells were induced with 1mM of IPTG, in LB medium, at 37°C and analyzed by Western blot using an anti-His antibody as probe (Fig. 19). It were detected two bands, a strong one with a molecular mass of 35 kDa and another one of around 75 kDa, in cells harbouring both constructions, but not a band of ≈65 kDa, corresponding to Gup1p (Fig. 19).

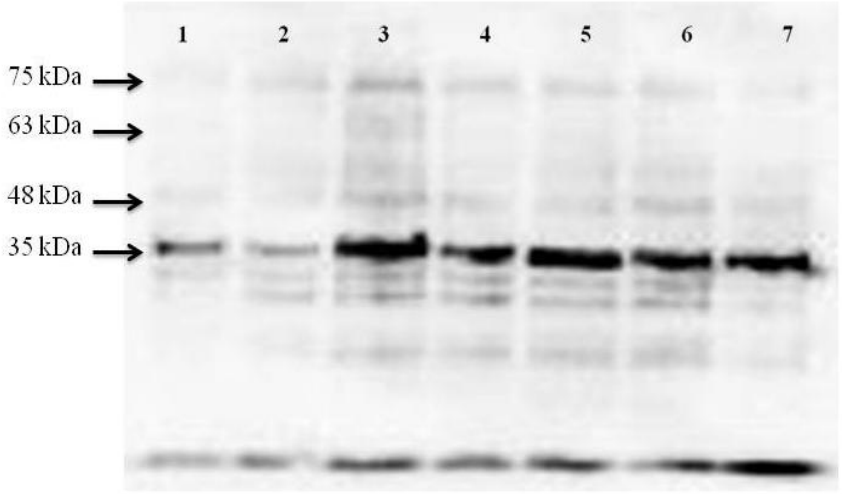

Figure 19 Western blot analysis using anti-His antibody (1:3000) of *E.coli* cells grown in LB media at 37°C with 1mM of IPTG, harbouring pET25 empty plasmid – column 1, pET25b-*GUP1* - column 2 to 4, and pET29a-*GUP1* - column 6 and 7.

- **Influence of other parameters**

In order to detect the expression of Gup1p, alterations in other methodological parameters were considered, namely the treatment of samples before being loaded on the SDS-Page gel was modified. In that sense, β-Mercaptoethanol was used instead of Dithiothreitol (DTT). β-Mercaptoethanol is utilized as a reducing agent to prevent the oxidation of proteins, likewise DTT is also a strong reducing agent but becomes less potent as lower pH (Lukesh, *et al.,* 2012). Additionally, also a change in culture conditions was made. Lactose was used in substitution of IPTG, since lactose is an analogue of IPTG and operates in the same fashion over *lac* operon (Neubauer, *et al.*, 1992). This kind of induction was used with success for expression of the soluble NifA protein domains of *Herbaspirillum seropedicae* in *E. coli* (Monteiro, *et al.*, 2000). In this study, the authors compared the efficiency of the lactose and IPTG and showed that induction with lactose promoted a significantly higher percentage of these proteins in the soluble fraction, than when IPTG was used (Monteiro, *et al.*, 2000).

Thus, *E. coli* pET-25b-*GUP1* cells were grown: (i) in LB medium with glucose and inducted with 1mM of IPTG; and (ii) in LB medium with lactose overnight (Fig. 20). As controls *E.coli* BL21(DE3) wild type cells and cells harbouring pET25b empty plasmid were subject to the same treatment and conditions (Fig. 20). Yet, the modifications above mentioned did not result on an altered Gup1 protein expression (Fig 20). In fact, the band profile observed for the controls (wild-type cells (Fig.20 - column 6), cells harbouring the empty plasmid (Fig.20 - column 1), and cell with 0h induction (Fig.20 - column 2)) was apparently the same as the one displayed by cells harbouring the constructions and induced either by IPTG (Fig.20 - column 3 to 5) or by lactose (Fig.20 – column 7).

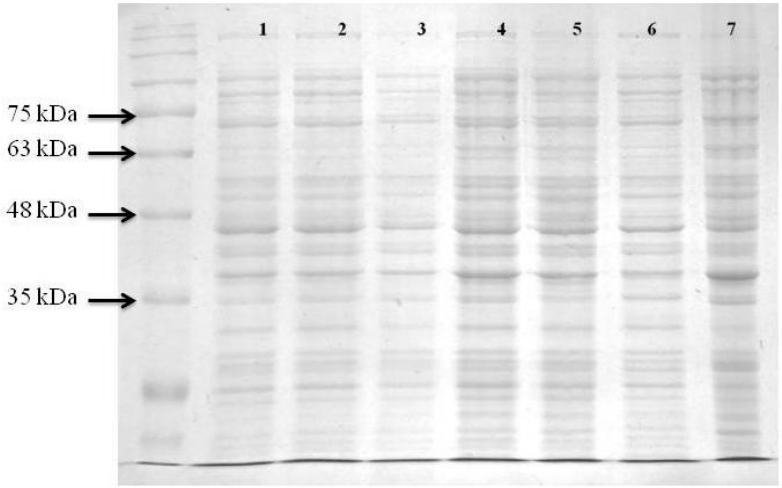

Figure 20 SDS-Page electrophoresis of *E.coli* BL21 (DE3) grown on LB medium at 37°C harbouring: empty plasmid pET25b - column 1; harbouring pET-25b-*GUP1* construction, induced with 0.1mM of IPTG for 2, 4 and 6 hours - column 2 to 5; and *E.coli* BL21 (DE3) wild-type - column 6; and harbouring pET-25b-*GUP1* grown in lactose 2% - column 7.

- **Influence of the temperature**

Lower culture temperatures may enhance functional protein formation, by reducing the rate at which an over-expression protein is formed. A reduced expression rate diminishes the concentration of the unfolded intermediates, which in turn may allow the proteins to preferentially follow the folding pathways rather than those leading to aggregation (Knappik, *et al.*, 1993). Thus, a lower cultivation temperature was assessed in *E. coli* cells harbouring the pET25b-*GUP1* and pET29b-*GUP1* constructions, which were grown at 18°C during 22 hours. Then, these were induced with 1mM of IPTG and the samples collected after 4, 6 and 22 hours. The samples of

all induction times were compared with the controls: *E.coli* BL21 (DE3) wild-type cells and the ones harbouring the empty plasmid, by SDS-Page. Having in mind, that once more was not observed any band that could correspond to the over-expressed Gup1 protein in none of the samples, despite the induction time (Fig. 21), it may be rationalized that for this particular case lowering the temperature did not cause any detected effect.

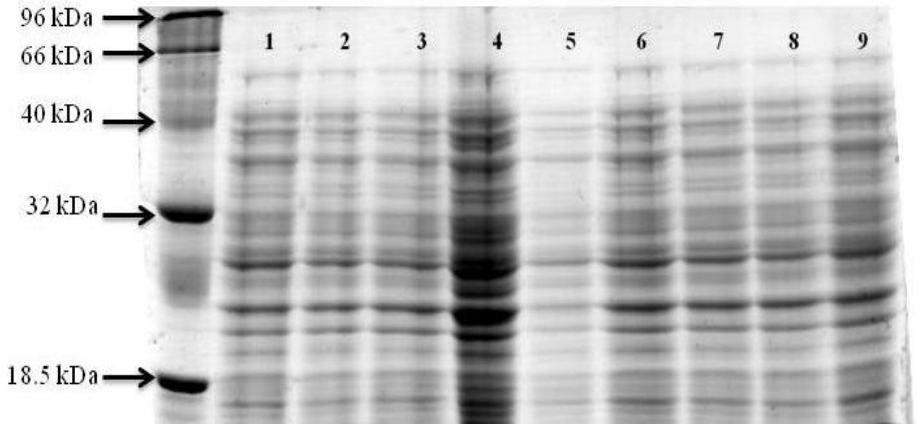

Figure 21 SDS-Page electrophoresis of *E.coli* BL21 (DE3) grown on LB medium at 18°C harbouring: empty plasmid pET25b - column 1; pET-25b-*GUP1* construction at 4, 6 and 22 hours - column 2 to 4; *E.coli* BL21 (DE3) wild type – column 5; empty plasmid pET29b – column 6; and pET-29b-*GUP1* construction at 4, 6 and 22 hours – column 7 to 9.

It is acknowledge that conditions such as temperature, growth medium and inducer concentration may influence the expression level of a protein. Therefore, when assessing a heterologous expression of a yeast protein in *E. coli* system, (as it was the case of this study) the results obtained can be influenced by such parameters. We evaluate some of these parameters in our attempt to express *S. cerevisiae* Gup1p in *E.coli* BL21 (DE3) using pET-25b and pET-29b expression systems, yet, we were not able to detect Gup1p expression. This either by SDS-Page analysis or by Western blots. The reasons underlying such event are not clear, however, other unsuccessfully attempts to express Gup1p (or just some peptides from this protein) using several expression systems and conditions, have been reported (Bleve G., PhD thesis; Ferreira C., PhD thesis). In one specific experiment other cultivation temperature was also tried, *E.coli* BL21-CodonPlus-RIL cells harbouring pET-30a-*GUP1* were grown at 30 and 37°C and induced with 0.1 mM and 1 mM of IPTG, and once more the expression of Gup1p failed (Bleve PhD thesis). Likewise, other experiment putative cytosolic and extracellular membranes domains of Gup1p were selected as possible antigens for

antibody production against Gup1 proteins. These domains were cloned in three different expression plasmid pET-14, pET-24a and pET-42 into *E. coli* BL21, Rosetta2 (DE3) and BL21-CodonPlus-RIL cells. These attempts were as well unsuccessfully (Ferreira, C., PhD thesis). The same author used, synthetic peptides as an alternative, yet the obtained crude antiserum turned out to be very unspecific for detection of Gup1 protein (Ferreira, C., PhD thesis).

3.2. Expression in *Saccharomyces cerevisiae Δgup1 strain*

3.2.1. Expression of *S.cerevisiae* Δ*gup1* using Gup1p-His-tag plasmid construction

The primary approach to express the Gup1 protein in yeast was performed by cloning the *GUP1* into two different vectors: (i) the pYES3/CT vector, which is commercially available and includes a 6xHis tag, and (ii) pYES2 vector, to which we add the 6xHis tag coding sequence, to the 3'-end region of the *GUP1* gene, before cloning. Both vectors contain the *GAL1* promoter for high level inducible protein expression in yeast by galactose and repression by glucose. *S. cerevisiae Δgup1* cells harbouring both constructions were grown in YNB media for 4 hours and then induced with galactose (transferred for YNB media with 2% of galactose).

An initial time course experiment was performed to determine the expression window of the recombinant protein. The cells were collected and tested for expression after 0, 4, 6 and 8 hours of galactose induction. Total protein extracts were prepared by TCA, and the precipitated proteins were separated by SDS-Page (10%) and further analyzed by Western blot. The membrane, after incubation with anti-HIS antibody (1:3000) overnight and with the secondary anti-mouse antibody (1:10000) for 1 hour, did not presented any reactive band (data not shown). Yet, Bleve G. with the same expression system (pYES2-*GUP1*) and using these same conditions was able to express this protein, though in a very low yield (15-20 ng/ml) (Bleve G., PhD thesis). The author concluded that the homologous expression in *S.cerevisiae* strains was not suitable for a large scale production of Gup1 (Bleve G., PhD thesis). It is possible, though not very probably, that the in our experiments the system is in fact expressing Gup1p, but in amounts that are below the Western blot sensitivity cutoff.

3.2.2. Expression of *S. cerevisiae Δgup1* using Gup1p-GFP fusion

Bleve and co-workers have cloned *GUP1* cDNA into a multicopy expression vector tagged with GFP at its carboxy- and amino-terminus, and by fluorescent and electron microscope, as well as by confocal laser scanning microscopy, verified its expression in *S. cerevisiae Δgup1* strain (Bleve *et al.*, 2005; 2011). These authors (Francesco Grieco and Gianluca Bleve from the Instituto di Scienze Delle Produzioni Alimentari del Consiglio Nazionale Delle Ricerche (ISPA), Leece, Italy), collaborators of our group, provided us with the C-terminal construction pYES2-*GUP1*-GFP plasmid. The first step was then to verify this construction by electrophoresis using the *HindIII* and *XhoI* restriction enzymes (Fig. 22). The expected bands were detected, one of ≈6000bp, corresponding to the plasmid size, and another one of ≈2500bp, corresponding to the chimera *GUP1*-GFP size (1700 bp of *GUP1* and 717 bp of *GFP*). Therefore, we transformed our own *S. cerevisiae* strain deleted in *GUP1* gene with such construction.

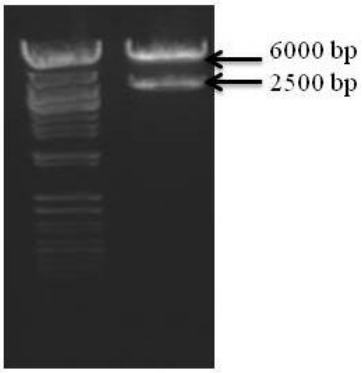

Figure 22 Electrophoretic analysis of pYES2-*GUP1*-GFP. Molecular weight marker Lambda DNA/Eco471, Marker 13 [Fermentas], - column 1. Double digestion with *HindIII* and *XhoI* of pYES2-*GUP1*-GFP ≈6000bp corresponding to the plasmid size and ≈2500bp corresponding to the chimera *GUP1*-GFP (1700 bp of *GUP1* and 717bp, of *GFP*), - column 2.

3.2.3. Localization of *GUP1*-GFP fusion protein

The localization of Gup1-GFP in *S.cerevisiae* Δ*gup1 ScGUP1*-GFP was inspected by fluorescence microscopy (Fig. 23). For that the cells were grown in YNB media overnight and then induced with galactose 2%, over the period of 8 hours. The fluorescence signal started to be detected on the second induction hour, mainly localized at the level of the plasma membrane (Fig. 23 - panel I). As the period of incubation was rising, the fluorescence signal was gradually being localized at the plasma membrane but also at endoplasmic reticulum, with a maximum intensity at 4-6

hours (Fig. 23- panel II and III). Our results, showing the subcellular localization of Gup1p at the plasma membrane and the endoplasmic reticulum under derepressed conditions are in agreement with the ones obtained by Bleve and collaborators (Bleve, *et al.*, 2005). Also according to this authors when the culture is shifted to glucose-based media, Gup1p is targeted for degradation in the vacuole by endocytosis (Bleve, *et al.*, 2005). One the other hand, Holst and collaborators (Holst, *et al.*, 2000) described a more broad Gup1p subcellular localization, at the plasma membrane and the endoplasmic reticulum but also at the mitochondrial membrane. This multi-subcellular localization was based on subcellular fractionation by sucrose gradient and immunofluorescence detection (Holst, *et al.*, 2000). This approach can justify such diversified localization, once subcellular fractionation methodologies are most of the times tricky and prone of contaminations.

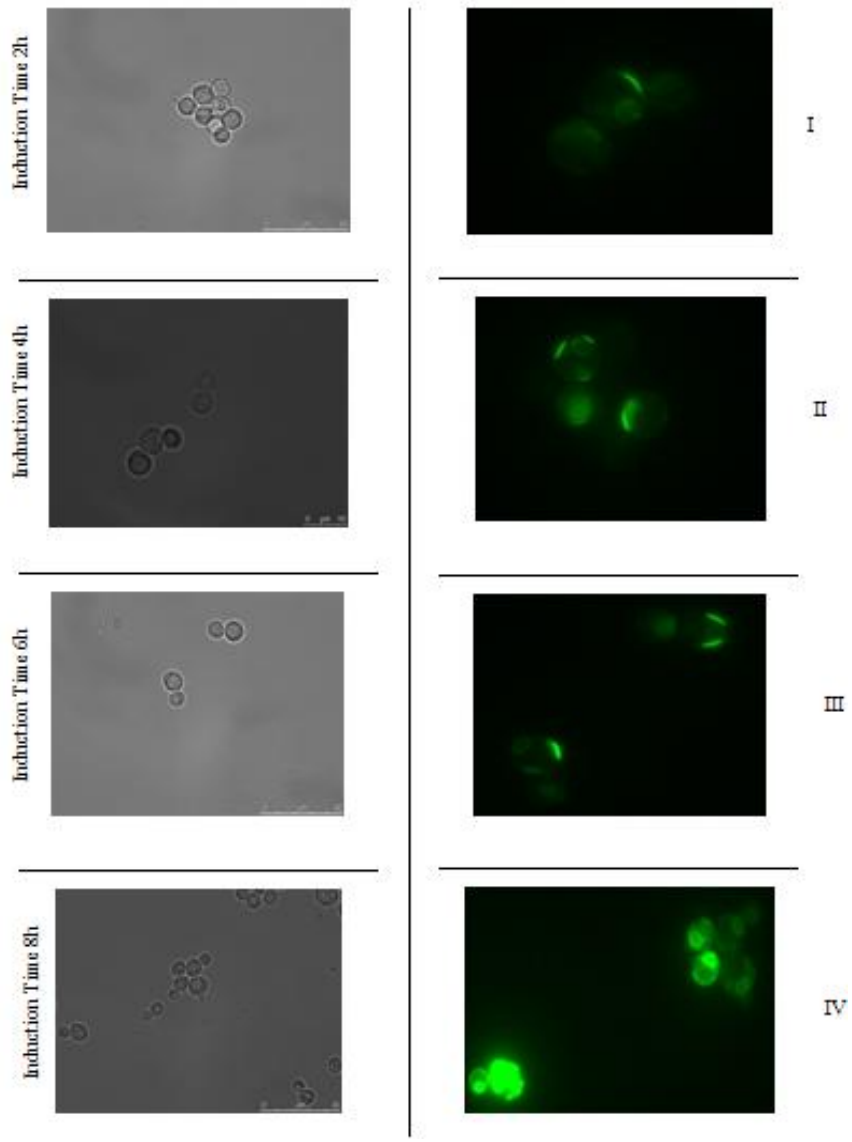

Figure 23 Localization of Gup1p-GFP fusion protein by fluorescence microscopy analysis of *S.cerevisiae Δgup1* strain transformed with pYES2-*GUP1*-GFP after 2, 4 6 and 8 hours of induction with galactose.

3.2.4. Expression of Gup1-GFP fusion protein in *S. cerevisiae Δgup1*

We used the same construction pYES2-*GUP1*-GFP to study the expression of Gup1p by Western blot analysis, using anti-GFP antibody. Five independent clones were grown overnight in YNB medium with glucose as carbon source. Then these were transferred and diluted to YNB media with galactose 2%, OD_{600} of ≈0.4, and incubated at 30ºC for several periods of time. A Western blot was performed probing the total extracts with the anti-GFP serum (1:5000) (Fig. 24). Clones 2 to 5 displayed a reactive band of 90 kDa, being clone 5 the one that showed the most intense band (after 4 hours of induction), reflecting a higher expression of Gup1p-GFP (Fig. 24A).

In a second similar experiment, this clone (clone 5) was induced over a longer period, 24 hours. In this, the expression of *GUP1*-GFP was detected in low amounts during the first four hours of induction, mirrored by a faint reactive band of the expected size, 95 kDa band (Fig 24B). This expression, as well as the intensity of the band increased along with the induction period; attaining a maximum at 24h of induction (Fig 24B). Yet, at this time point, other unspecific bands (140 kDa, 75 kDa, 30 kDa and 40 kDa) were visualized (Fig. 24B). The same cells were further inspected by fluorescence microscopy, displaying a maximum fluorescence after 4-6 hours of induction (Fig 24C).

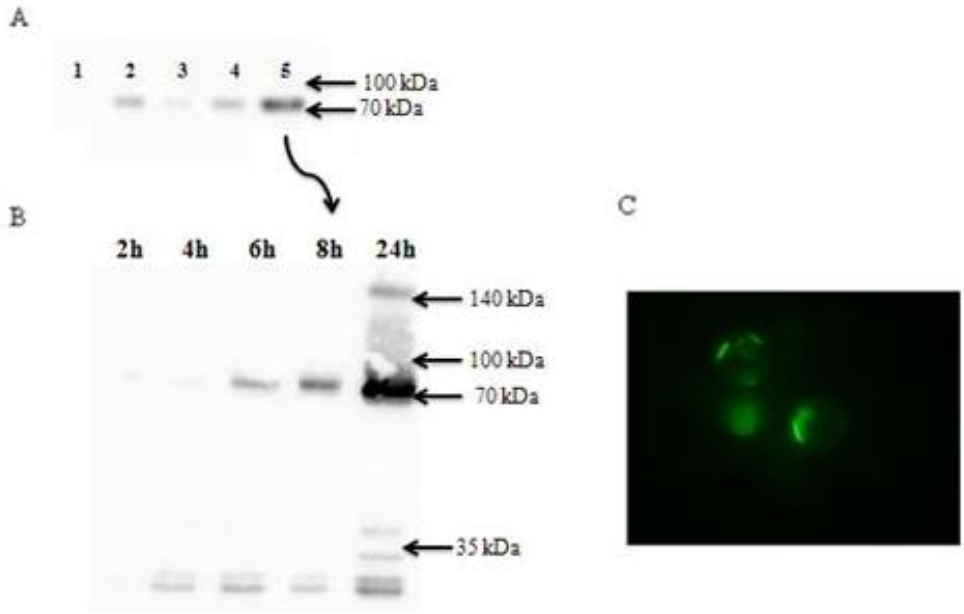

Figure 24 Expression of five different clones of *S. cerevisiae Δgup1* harbouring pYES2-*GUP1*-GFP (A). Expression of the clone 5 after 2, 4, 6, 8 and 24 hours of induction (B); Fluorescent microscopy representative photo of *S. cerevisiae Δgup1* harbouring pYES2-*GUP1*-GFP after 4 to 6 hours induction (C).

3.2.5. Immunoprecipitation

Immunoprecipitation is a methodology commonly used for a variety of purposes, protein expression, purification, and subcellular localization. With this technique, the antigen is isolated by binding a specific antibody. Antibodies, in turn bound noncovalently immunoadsorbents such as protein A– or protein G–agarose, or can be coupled covalently to a solid-phase matrix. Immunoprecipitation protocols consist of several stages. Importantly, when applied to yeast and because yeasts have an extremely resistant and detergent-insoluble cell wall, which is composed mainly by

mannoproteins, chitin, and glucans (Ferreira, *et al.,* 2006), this method requires an initial step. This step consists in disrupting the cell wall, by mechanical, enzymatic, or chemical procedures, in order to allow an efficient extraction of the antigens in the subsequent stages. The most frequently procedure consists of vigorous vortexing of the yeast suspension with glass beads. The breakage can be done in the presence or absence of detergent as detergents play a significant role in this effort. They serve as tools to isolate, solubilise and manipulate membrane proteins to subsequent biochemical and physical characterization (Garavito, *et al.,* 2001). Therefore, the choices of detergent and of the experimental conditions are parameters to have into account, when a membrane protein is the target, as is the case of Gup1p.

Hence, the immunoprecipitation assay was performed with *S.cerevisiae Δgup1* harbouring the fusion protein Gup1p-GFP, the same strain harbouring the pYES2 empty plasmid (named control strain). The cells were grown overnight, at 30°C in YNB media with glucose as carbon and energy source. The induction was performed with galactose 2%, during 4 hours. Then, the immunoprecipitation protocol was followed, being obtained the two distinct set of samples: Samples A - suspension of proteins obtained in the end of the immunoprecipitation protocol, ii) Sample B- same as A but the step of adding anti-GFP antibody was removed, meaning that these samples were directly incubated with the protein G-Sepharose beads.

Thus, *Δgup1* strain harbouring Gup1p-GFP and the control strain were subjected to lysis with glass beads, and then incubated with the immunoprecipitation buffer, containing NP-40 detergent, to solubilise the protein in non-denaturising conditions. After that, these samples were incubated with anti-GFP antibody overnight, followed by the addition of the protein G-Sepharose. The obtained suspension - Samples A - were analyzed by SDS-Page (10%) (Fig. 25) and by Western blot with anti-GFP antibody (data not shown). On the SDS-gel, was possible to detect a band of about 43 kDa (Fig 25 - column 2, right arrow) that was not visible on the control (Fig. 25 - column 1). On the other hand, the Western blot did not present any reactive band (data not shown). These may due to (i) an inefficient solubilisation process, or (ii) to the use of a low amount of initial culture. In this study, only 15ml of culture were used to perform the assay and that probably was not enough to obtain a reasonable amount of protein. Conversely, only one detergent was assessed, the NP40, and this may not be the best choice to solubilise our protein. At this point, and considering that the protein

is being expressed as suggested by the results on section 4.4, further tests should be performed, using higher quantities of initial culture and testing other detergents (*e.g.* Tween 20, Triton-X, Cetyl methyl ammonium bromide (CTAB), CHAPS, etc.).

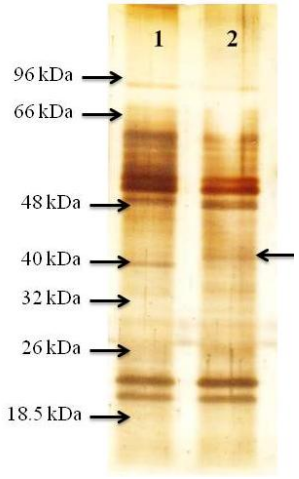

Figure 25 Immunprecipitation SDS gel stained with silver of the Sample A. Column 1-control.

Samples B, as mentioned above, were obtained from the protocol missing the anti-GFP antibody step. These were also analyzed by SDS-Page and by Western blot (data not shown), being the result similar to the one obtained with Samples A.

Faced with these results, it was decided to verify if those were due to an absence of protein expression, or due to protocol difficulties. Therefore, the same strains were grown and induced in equal conditions and then subjected to lysis with glass beads. The suspension obtained after removing the beads - lysate- was precipitated with TCA, separated by SDS-Page (10%) (data not shown) and further analyzed by Western blot (Fig. 26). The SDS-Page profile was identical to the one obtained for Sample A (Fig. 25 - column 2), whereas the Western blot revealed a reactive band of about 90 kDa, not present in the control strain (Fig. 26- column 2 and 1, respectively). Thus, it seems that the protein is being expressed, this is the expected size and is in agreement with the results of Bleve and co-workers that also detected a 95 kDa band deriving from the expression of Gup1p-GFP fusion protein in *S. cerevisiae Δgup1* on the same conditions (Bleve, *et al.*, 2005). All taken, we have to consider that the problem might very well be on the solubilisation step. As mentioned before, the solubilisation of plasma membrane proteins is problematic and requires several optimization steps that due to the lack of time were not tested in the present work.

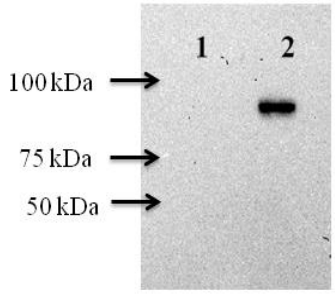

Figure 26 Western Blot analyses of the lysates, control strain (1) and strain harbouring Gup1p-GFP chimera (2).

Conclusions and Future Work

In order to be able to express Gup1 protein, ultimately in enough amounts to eventually obtain as well its molecular partners, a set of *S. cerevisiae* and *E.coli* strains harbouring different expression plasmids/systems were constructed within this work.

We exhaustively tested the Gup1p expression in *E. coli* BL21(DE3)-CodonPlus-RIL using two different plasmids, pET25b and pET29b, and despite of the high number of conditions and optimizations made, the expression was never attained. Even being this strain considered the best vehicle for heterologous protein expression, the system, within this work, revealed itself not to be suitable for the expression of Gup1p, as it wasn't, as well, in previous studies (Ferreira, C., PhD Thesis 2005; Bleve, G., PhD Thesis 2005).

The homologous expression in *S. cerevisiae Δgup1* mutant with pYES2 and pYES3 plasmids harbouring the *GUP1* gene tagged to histidines tag was also attempted, under several conditions. Although, previously this system (pYES2) worked to expressed Gup1p, even that in very low amounts (Bleve, G., PhD Thesis 2005), we were not succeeded. Whereas, this also does not seem to be an efficient system to express Gup1p, at least in large amounts.

Conversely, the expression of Gup1p using a chimera with GFP reporter protein was detected, both by Western blot assay and by fluorescent microscopy. These experiments also allowed to confirm the previously reported subcellular localization of Gup1p, mainly in plasma membrane and endoplasmic reticulum.

Having a system in which was attained the expression of Gup1, this was further used on immunoprecipitation assays, to purify Gup1p and potentially some partners. This assay is still being improved, as in the first attempts Gup1p was not retained by the protein G-Sepharose beads. In our opinion this may be due to (i) small amount of initial culture, which may not be enough for the detection of the protein by Western blot, and (ii) the detergent used to solublize the protein was not the appropriated one, once that when we test the lysates the protein was detected. In that sense, in the subsequent immunoprecipitation assays higher quantities of initial culture should be used, but also other detergents have to be tested. Alternatively, it could be considered the construction of an affinity anti-GFP column. Yet, by one side the size of GFP

protein may be an issue for the purification of Gup1 protein, whereas GFP also is known to bind several nonspecific products.

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