Chapter 5

Role of PI-3 kinase in pathogenesis of *C. glabrata*
5.1 Introduction

*C. glabrata* cells, upon macrophage engulfment, undergo metabolic and cellular energy reprogramming to survive and replicate in host macrophages (Kaur *et al*., 2007; Seider *et al*., 2011). Genome-wide transcriptional analyses revealed that genes encoding enzymes involved in β-oxidation, gluconeogenesis and glyoxylate cycle were induced in *C. glabrata* cells in response to macrophage internal milieu (Kaur *et al*., 2007; Seider *et al*., 2011). Importantly, autophagy and pexophagy have been shown to play important roles in redistribution and mobilization of stored cellular metabolites and help *C. glabrata* cells survive in nutrient-deprived host cells (Roetzer *et al*., 2010).

In Chapter 3, we discussed the identification of a set of 56 genes belonging to diverse cellular processes including chromatin organization, which were required by *C. glabrata* for its survival and/or replication in THP-1 macrophages. Ten of the identified mutants displaying diminished survival had Tn7 insertion in genes implicated in intracellular vesicular transport. Genes involved in vesicular trafficking and their molecular functions have been described in Table 5.1. Of these ten genes, we decided to first focus on *CgVPS15* (*CAGL0H08437g*) which codes for a putative membrane-associated serine/threonine protein kinase (http://genolevures.org/cagl.html#). *S. cerevisiae* ortholog of *CgVPS15* encodes the regulatory subunit, Vps15, of phosphatidylinositol-3 (PI-3) kinase (Stack *et al*., 1995a). In *S. cerevisiae*, PI-3 kinase is composed of two subunits, namely, regulatory subunit Vps15 and catalytic subunit Vps34 (Stack *et al*., 1995a). PI-3 kinase is a lipid kinase, which phosphorylates third hydroxyl group of phosphatidylinositol to produce phosphatidylinositol-3-phosphate (Herman *et al*., 1991). PI-3 kinase is one of the key regulators of vesicle-mediated transport and inositol lipid-mediated signaling (Herman *et al*., 1991; Stack *et al*., 1993). Importantly, Vps15 and Vps34 are also required for Gpa1 (GTP-binding alpha subunit of the heterotrimeric G protein)-mediated pheromone signaling at the endosome in *S. cerevisiae* (Slessareva *et al*., 2006). In the current chapter, we describe experiments performed to investigate the role of PI-3 kinase and G protein-coupled receptor (GPCR)-mediated signaling in pathogenesis of *C. glabrata*.
Table 5.1: List of the vesicular trafficking mutants identified in STM-screen

<table>
<thead>
<tr>
<th>Mutant</th>
<th>CAGL-ORF</th>
<th>S. cerevisiae ortholog</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Golgi vesicle transport</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cgerv29::Tn7</td>
<td>CAGL0G00176g</td>
<td>ERV29</td>
<td>Protein localized to COPII-coated vesicles</td>
</tr>
<tr>
<td>Cggea2::Tn7</td>
<td>CAGL0M08052g</td>
<td>GEA2</td>
<td>Guanine nucleotide exchange factor for ADP ribosylation factors</td>
</tr>
<tr>
<td>Cgpho86::Tn7</td>
<td>CAGL0L05456g</td>
<td>PHO86</td>
<td>Endoplasmic reticulum resident protein</td>
</tr>
<tr>
<td>Cger1::Tn7</td>
<td>CAGL0C01837g</td>
<td>RER1</td>
<td>Protein involved in retention of membrane proteins, including Sec12, in the ER</td>
</tr>
<tr>
<td>Cgsro7::Tn7</td>
<td>CAGL0K05291g</td>
<td>SRO7</td>
<td>Effector of RabGTPase Sec4</td>
</tr>
<tr>
<td><strong>Endocytosis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cgact1::Tn7</td>
<td>CAGL0K12694g</td>
<td>ACT1</td>
<td>Actin, structural protein</td>
</tr>
<tr>
<td>Cgldb17::Tn7</td>
<td>CAGL0J03696g</td>
<td>LDB17</td>
<td>Protein of unknown function</td>
</tr>
<tr>
<td>Cgpan1::Tn7</td>
<td>CAGL0J01892g</td>
<td>PAN1</td>
<td>Part of actin cytoskeleton-regulatory complex Pan1-Sla1-End3</td>
</tr>
<tr>
<td>Cgsla2::Tn7</td>
<td>CAGL0J07656g</td>
<td>SLA2</td>
<td>Transmembrane actin-binding protein</td>
</tr>
<tr>
<td><strong>Signaling</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cgvps15::Tn7</td>
<td>CAGL0H08437g</td>
<td>VPS15</td>
<td>Myristoylated serine/threonine protein kinase involved in vacuolar protein sorting</td>
</tr>
</tbody>
</table>

5.2 Results

5.2.1 PI-3 kinase mutant, Cgvps15, is impaired in preventing phagolysosomal acidification and survival in macrophages

*C. glabrata* cells survive and replicate in host macrophages and are capable of preventing phagolysosomal acidification (Figure 5, Chapter 3). To examine the role of intracellular vesicular trafficking in pathogenesis of *C. glabrata*, we first sought to examine the ability of mutants defective in vesicle-mediated transport to survive/replicate in macrophages and prevent phagolysosomal acidification. In our
STM screen for altered survival profiles in THP-1 macrophages, we identified ten mutants, Cgerv29, Cggea2, Cgpho86, Cgrer1, Cgsro7, Cgact1, Cgldb17, Cgpan1, Cgsla2 and Cgvps15, which carried Tn7 insertions in genes implicated in intracellular trafficking. Since STM screen was performed in a pool-wise fashion wherein survival of 96 mutants was assessed at once in a single infection experiment, we decided to validate diminished survival of these mutants in THP-1 macrophages by single infection assay. In brief, we infected Cgerv29, Cggea2, Cgpho86, Cgrer1, Cgsro7, Cgact1, Cgldb17, Cgpan1, Cgsla2 and Cgvps15 mutants individually to PMA-differentiated THP-1 macrophages at a MOI of 1:10 (yeast: macrophage). Post 24 h coincubation, macrophages were lysed and survival of vesicular trafficking mutants was assessed by colony forming unit (CFU) assay. Cgerv29, Cggea2, Cgpho86, Cgact1, Cgldb17 and Cgsla2 mutants displayed survival ratio of ~ 0.3 to 0.6 (Figure 5.1A). Intriguingly, Cgrer1 and Cgsro7 mutants did not exhibit any significant defect in survival in THP-1 macrophages. In contrast, only 1-3% of Cgpan1 and Cgvps15 mutant cells were found to be viable after 24 h coculturing with macrophages (Figure 5.1A).

Next, we examined the trafficking mutants for their ability to prevent phagolysosomal maturation by confocal fluorescence microscopy. GFP-expressing wild-type and fluorescein isothiocyanate (FITC)-labeled trafficking mutants were infected to PMA-differentiated THP-1 macrophages and acidified phagolysosomes were visualized by lysotracker staining after 2 h coincubation. Cgerv29, Cggea2, Cgpho86, Cgact1, Cgldb17 and Cgsla2 mutants displayed only 1-6% colocalization of FITC and lysotracker similar to wild-type-infected macrophages (Figure 5.1B). Surprisingly, in Cgvps15-infected macrophages, approximately 16% colocalization of FITC and lysotracker was observed indicating that Cgvps15 cells were probably deficient in preventing acidification of phagolysosome in THP-1 cells (Figure 5.1B). As discussed in Chapter 3, wild-type C. glabrata cells were able to modulate phagolysosomal acidification and colocalization of yeast cells with acidified lysosome (lysotracker-positive compartment) was observed only when C. glabrata cells were heat-killed at 95°C prior to THP-1 infection. To preclude the possibility that ~15% of Cgvps15 mutant cells which colocalize with lysotracker are not dead, we determined the number of viable yeast cells at early time points post infection viz., 0, 1, 2, and 4 h by trypan blue
staining but observed no appreciable cell death till 4 h post infection. These results indicate that diminished survival of Cgyps15 mutant in THP-1 macrophages may partly be attributed to its inability to modulate phagolysosomal acidification.

**Figure 5.1.** *C. glabrata* PI-3 kinase mutant display impaired modulation of phagolysosomal acidification and reduced survival in macrophages. (A) PMA-differentiated THP-1 macrophages were infected with indicated strains. Post 24 h coincubation, viability of intracellular yeast cells was determined by CFU assay as discussed in Chapter 3. Survival ratio indicates the ratio of fold replication of mutant to that of wild-type cells 24 h post infection. (B) Intracellular trafficking mutants were FITC-labeled and infected to THP-1 macrophages. After 2 h coincubation, macrophages were stained with Lysotracker-red. Percentage colocalization was determined by dividing the number of lysotracker positive cells by that of FITC positive cells. Data represent the mean ± standard deviation from three independent experiments.
5.2.2 Generation of Cgvp515Δ, Cgvp34Δ and Cggpa1Δ strains

In S. cerevisiae, Vps15 acts as a regulatory subunit of PI-3 kinase, which phosphorylates and activates the catalytic subunit Vps34 (Stack et al., 1995a). To investigate the molecular role of PI-3 kinase in pathogenesis of C. glabrata, we deleted the ORFs, CAGL0H08437g and CAGL0G08360g, which code for CgVps15 and CgVps34, respectively, from the C. glabrata genome. CgVps15 and CgVps34 share 44 and 63% amino acid homology with their S. cerevisiae counterparts. As PI-3 kinase is known to interact with trimeric G-protein complex (Slessareva et al., 2006), we sought to examine if survival and proliferation of C. glabrata in macrophages is dependent on GPCR-mediated signaling. For this, we deleted the ORF CAGL0F06677g which codes for CgGpa1, GTP-binding alpha subunit of the heterotrimeric G protein. CgGpa1 displayed 69% amino acid similarity with ScGpa1. We utilized fusion PCR-based method to construct Cgvp515Δ, Cgvp34Δ and Cggpa1Δ deletion strains. In brief, approximately 1 kb 5’ and 3’ UTRs of the gene of interest were amplified and fused with two nat1 halves by fusion PCR. PCR products carrying 5’ and 3’ UTRs fused with first and second half of nat1, respectively, were used to transform wild-type C. glabrata cells in uracil prototrophic and auxotrophic backgrounds. Replacement of the desired gene with nat1 cassette was confirmed by PCR using primers to confirm 5’ and 3’ homologous recombination (Figure 5.2).

To investigate whether these deletion strains are attenuated for growth in THP-1 macrophages, we first examined the ability of Cgvp515Δ and Cgvp34Δ mutants to prevent phagolysosomal acidification. Similar to Cgvp515 insertion mutant, Cgvp515Δ and Cgvp34Δ deletion mutants displayed increased colocalization with lysotracker dye in THP-1 macrophages compared to the wild-type cells after 2 h infection (Figure 5.3). Further, to examine if the inability of Cgvp515Δ and Cgvp34Δ mutants to prevent phagolysosomal maturation led to altered survival in host macrophages, we infected PMA-differentiated THP-1 macrophages with Cgvp515Δ and Cgvp34Δ cells and determined their survival and/or replication after 24 h coincubation. Cgvp515Δ and Cgvp34Δ mutants were killed by THP-1 macrophages and displayed only 1-3% viability after 24 h of coincubation, while wild-type cells underwent 5- to 6-fold replication during the same period (Figure 5.4A). Notably, Cggpa1Δ cells displayed wild-type-like behavior in response to macrophage phagocytosis. During 24 h coincubation with THP-1 macrophages, Cggpa1Δ cells
underwent ~ 5-fold replication suggesting that probably GPCR-mediated signaling is not essential for survival of *C. glabrata* cells in macrophages (Figure 5.4A).

**Figure 5.2.** Confirmation of *CgVPS15, CgVPS34* and *CgGPA1* disruption in *Cgvs15Δ, Cgvs34Δ* and *Cgpa1Δ* deletion strains. Genomic DNA from the deletion mutants and wild-type strains were extracted and disruption of desired ORFs was confirmed using primers for 5’ (1) and 3’ (2) homologous recombination and gene internal region amplification. Figures A, B and C confirm the deletion of *CgVPS15, CgVPS34* and *CgGPA1* ORFs, respectively.
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**Figure 5.3.** *C. glabrata* PI-3 kinase deletion mutants are impaired in modulating phagolysosomal acidification. GFP-expressing *CgVps15Δ* and *CgVps34Δ* cells were infected to PMA-activated THP-1 macrophages. Macrophages were stained with Lysotracker-red and examined for colocalization of green and red signal under Carl-Zeiss LSM510 metaconfocal microscope. Scale bar = 10 µm.

To corroborate the above findings, we also examined survival of these mutants in primary murine macrophages derived from BALB/c mice. Consistent with their diminished survival in human THP-1 macrophages, only 1-2% of *CgVps15Δ* and *CgVps34Δ* cells were found to be viable post 24 h coinoculation with primary macrophages (Figure 5.4B). Notably wild-type cells showed ~ 6-fold replication in primary murine macrophages (Figure 5.4B). Proliferation of *Cggpa1Δ* cells in primary murine macrophages was not examined as they did not exhibit any survival defect in THP-1 macrophages. Importantly, *CgVps15Δ* and *CgVps34Δ* strains expressing *CgVPS15* and *CgVPS34* genes ectopically from plasmids pRK942 and pRK984, respectively, showed wild-type-like proliferation in both THP-1 and primary murine macrophages *viz.* 5- to 6-fold (Figures 5.4A & B), indicating that viability loss of these mutants in macrophages is due to the lack of *CgVps15* and *CgVps34*, respectively. Collectively, these findings suggest that both catalytic and regulatory subunits of PI-3 kinase are essential in *C. glabrata* to modulate phagolysosomal acidification and proliferate in host macrophages.
Figure 5.4. *C. glabrata* PI-3 kinase deletion mutants are unable to survive in macrophages. PMA-treated THP-1 macrophages (A) and peritoneal macrophages (B) were infected with indicated strains to a MOI of 1:10 and fold replication was calculated after 24 h coincubation. Data represent the mean ± standard deviation from three independent experiments.

Further, to investigate if the inability of *Cgypsl5Δ* and *Cgyp34Δ* cells to survive in THP-1 and primary macrophages is associated with general growth defects and reduced proliferation in RPMI medium, we examined their growth in YPD and RPMI media at 30°C and cell culture conditions, respectively. Growth curve analyses
of wild-type, \textit{Cgyps15} and \textit{Cgyps34} mutants indicated that though PI-3 kinase mutants grew slowly compared to wild-type, they were able to reach significant cell densities in RPMI medium under tissue culture conditions (Figure 5.5). Collectively, these results suggested that PI-3 kinase in \textit{C. glabrata} plays a pivotal role in preventing phagolysosomal acidification and survival in host macrophages.

**Figure 5.5. Growth analysis of \textit{C. glabrata} strains in RPMI-1640 and YPD media.** Indicated \textit{C. glabrata} strains were inoculated in RPMI-1640 (A) and YPD media (B) to an initial OD$_{600}$ of 0.2 and 0.1, respectively, and their growth was monitored till 48 h at regular time intervals.

**5.2.3 Killing of \textit{Cgyps15} and \textit{Cgyps34} mutants in macrophages is independent of reactive oxygen species (ROS) generation**

Macrophages are known to produce reactive oxygen species (ROS) and pose adverse conditions for pathogens to survive intracellularly (Forman and Torres, 2001). Wild-type \textit{C. glabrata} cells prevent macrophage-mediated generation of reactive
oxygen species for their survival and/or proliferation (Wellington et al., 2009). Here, we investigated if diminished viability of PI-3 kinase defective mutants in THP-1 macrophages is due to their inability to cope up with macrophage-generated reactive oxygen species. For this, first, we performed liquid growth assay to test the sensitivity of Cgyps15Δ and Cgyps34Δ towards oxidative stress. Time course analysis revealed attenuated growth of Cgyps mutants in YPD medium containing H2O2 during logarithmic phase (Figure 5.6A). Next, we used an inhibitor of NADH oxidase, diphenyleneiodinium (DPI). Notably, NADH oxidase is known to catalyze generation of reactive oxygen species in macrophages (Forman and Torres, 2001). We infected Cgyps15Δ and Cgyps34Δ cells to DPI-treated THP-1 macrophages to a MOI of 1:10 and determined their viability after 24 h coincubation with macrophages. CFUs obtained post 24 h infection indicated that inhibition of ROS generation in THP-1 macrophages could not reverse the survival defects of Cgyps15Δ and Cgyps34Δ cells (Figure 5.6B). This observation suggests that loss of viability of the Cgyps15Δ and Cgyps34Δ cells in macrophages may not be due to macrophage-generated ROS. Further, we examined if prolonged exposure of PI-3 kinase mutants to oxidative stress could lead to loss of their viability. For this, we treated Cgyps15Δ and Cgyps34Δ cells with a range (20 mM-100 mM) of H2O2 concentrations for four hours and determined the loss of viability by trypan blue staining. To our surprise, H2O2 treatment to Cgyps15Δ and Cgyps34Δ cells did not lead to enhanced cell killing, although significant proliferation was also not observed during this treatment.
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**A**

![Graph showing growth of different C. glabrata strains](image)

**B**

![Bar graph showing fold replication](image)

**Figure 5.6. Intracellular killing of Cgyps15Δ and Cgyps34Δ mutants in macrophages is independent of reactive oxygen species (ROS) generation.** (A) Overnight grown cultures of indicated strains were inoculated to an initial 0.1 OD₆₀₀ and their growth was monitored in YPD medium containing H₂O₂ (20 mM) till 48 h at regular time intervals. (B) PMA-differentiated THP-1 macrophages were incubated in diphenyleneiodinium (DPI) for 2 h prior to *C. glabrata* infection. Indicated *C. glabrata* strains were infected to THP-1 macrophages to a MOI of 1:10 and survival/replication was determined by CFU assay after 24 h coincubation. Data represent mean ± SEM and mean ± standard deviation from three independent experiments in Figures 5.6 A and B, respectively.

Next, we investigated if PI-3 kinase mutants, *Cgyps15Δ* and *Cgyps34Δ*, induced altered immune response compared to wild-type cells, when infected to THP-1 macrophages. PMA-activated THP-1 macrophages were coincubated with wild-type and mutant cells for 24 h and levels of IL-4 and IL-6 cytokines were measured. *Cgyps34Δ* cells elicited 3- to 4-fold higher production of IL-4 and IL-6 in THP-1
macrophages compared to wild-type cells. Intriguingly, Cgps15Δ-infected THP-1 macrophages displayed 2- to 3-fold induction in IL-4 production, however, no significant change in levels of IL-6 was observed (Figure 5.7). Reasons for this differential activation of host cytokine response by Cgps15Δ and Cgps34Δ mutants are not clear and warrant further investigation.

![Figure 5.7. Cgps34Δ mutant elicits higher IL-4 and IL-6 production by THP-1 macrophages.](image)

**Figure 5.7. Cgps34Δ mutant elicits higher IL-4 and IL-6 production by THP-1 macrophages.** Indicated *C. glabrata* strains were infected to PMA-differentiated THP-1 cells to a MOI of 1:10. After 24 h coincubation, supernatants were collected and cytokines were measured using BD OptEIA ELISA kits following manufacturer’s instructions. Data represent mean ±SEM from three independent experiments.

### 5.2.4 Cgps15Δ and Cgps34Δ mutants display increased sensitivity towards oxidative, cell wall and thermal stresses

In *S. cerevisiae*, PI-3 kinase is involved in diverse cellular processes (Stack *et al.*, 1995b). To investigate the effects of PI-3 kinase signaling disruption on cell physiology, we examined susceptibility of Cgps15Δ, Cgps34Δ and Cggpa1Δ mutants to different stress-causing agents. For this, *C. glabrata* cultures were spotted on YPD medium containing H₂O₂, NaCl, hydroxyurea, caffeine and menadione. Cgps15Δ and Cgps34Δ mutants displayed attenuated growth in presence of H₂O₂ and menadione suggesting that PI-3 kinase is required to survive the oxidative stress (Figure 5.8). Additionally, Cgps15Δ and Cgps34Δ cells exhibited elevated sensitivity towards caffeine while their growth remained unaffected in presence of NaCl (Figure 5.8). Notably, Cggpa1Δ cells grew similar to wild-type and did not show sensitivity towards any stress (Figure 5.8). Upon exposure to elevated temperature, Cgps15Δ and Cgps34Δ cells grew well at 37°C; however, their growth was severely compromised at 42°C (Figure 5.8). Intriguingly, Cgps15Δ and
Cgvsps34Δ mutants displayed diminished growth in presence of hydroxyurea (Figure 5.8). Growth defects of Cgvsps15Δ and Cgvsps34Δ mutants were rescued when corresponding ORFs were ectopically expressed from plasmids (Figure 5.8). Collectively, above findings suggest that PI-3 kinase is essential to survive oxidative, cell wall and thermal stresses in C. glabrata while GPCR-mediated signaling is not required for general stress tolerance.

Further, to investigate if CgVPS34 codes for a functional PI-3 kinase in C. glabrata, we assessed PI-3 kinase activity in wild-type and Cgvsps34Δ cells by in vitro PI-3 kinase assay (Whitman et al., 1988). In brief, sonicated phosphatidyl inositol was coincubated with cell lysates in the presence of γ-P32 ATP and radiolabelled phosphatidylinositol-3-phosphates (PI-3P) synthesized were quantified. As shown in Figure 5.9, Cgvsps34Δ cells displayed negligible amount of phosphatidylinositol-3-phosphate in comparison to wild-type cells indicating that deletion of CgVPS34 abolished PI-3 kinase activity in C. glabrata.

Figure 5.8. C. glabrata PI-3 kinase mutants displayed elevated sensitivity towards oxidative, cell wall and thermal stresses. C. glabrata cells from 1 ml overnight grown cultures of indicated strains were harvested, PBS washed and diluted to a final OD600 of 1. Cultures were 10-fold serially diluted in PBS and 3 µl of each dilution was spotted on YPD plates containing NaCl (500 mM), hydroxyurea (HU, 75 mM), caffeine (7.5 mM), H2O2 (20 mM) and menadione (100 µM). Images were captured after 2 days.
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Figure 5.9. Deletion of *CgVPS34* abolishes PI-3 kinase activity in *C. glabrata*. Overnight grown wild-type and *Cgyps34Δ* were reinoculated in YNB medium and grown for 4 h. Log-phase grown cells were harvested and cell lysates prepared. PI-3P synthesized by *in vitro* PI-3 kinase reaction was resolved on silica-60 plate by thin layer chromatography as described in Chapter 2.

5.2.5 *Cgyps15Δ* and *Cgyps34Δ* mutants exhibit enlarged vacuolar morphology and attenuated growth on non-fermentable carbon sources

‘VPS’ stands for vacuolar protein sorting and *S. cerevisiae vps* mutants were initially identified as mutants displaying either altered vacuolar morphology or carboxypeptidase Y secretion (Bankaitis *et al*., 1986). To investigate if deletion of *CgVPS15* and *CgVPS34* ORFs leads to altered vacuolar morphology, we labeled the vacuoles with a fluorescent dye FM4-64 and performed confocal fluorescence microscopy analysis. As shown in Figure 5.10A, *Cgyps15Δ* and *Cgyps34Δ* log-phase cells possessed enlarged vacuoles while wild-type cells had smaller vacuoles (Figure 5.10A). Notably, in 58 and 64% of *Cgyps15Δ* and *Cgyps34Δ* mutant cells, vacuoles occupied most of the cell volume. Vacuolar morphology defect was complemented when *CgVPS15* and *CgVPS34* ORFs were expressed from *C. glabrata* CEN-ARS plasmid from *PGK1* promoter (Figure 5.10A).
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![Diagram of vacuolar morphology and growth on non-fermentable carbon sources.](image)

**Figure 5.10.** *Cgups15Δ* and *Cgups34Δ* mutants exhibit enlarged vacuolar morphology and attenuated growth on non-fermentable carbon sources. (A) YPD-grown log-phase cells of indicated *C. glabrata* strains were labeled with FM 4-64 and observed under confocal microscope at 60X magnification. Scale bar represents 2 µm. (B) *C. glabrata* cells from 1 ml overnight grown cultures of indicated strains were harvested, PBS washed and diluted to a final OD$_{600}$ of 1. Cultures were 10-fold serially diluted in PBS and 4 µl of each dilution was spotted on YNB plates having dextrose (2%), ethanol (2%), glycerol (3%) and oleic acid (2%). Images were captured after 6 days.

In yeast, mutants with defective vacuolar functions are known to be impaired in their utilization of alternate carbon sources (van Voorst et al., 2006). To examine if *Cgups15Δ*, *Cgups34Δ* and *Cgga1Δ* cells can utilize non-fermentable carbon sources, we checked their ability to grow on non-fermentable carbon sources. Similar to *S. cerevisiae* PI-3 kinase mutants, *Cgups15Δ* and *Cgups34Δ* cells were unable to grow on YNB plates containing alternate carbon sources viz., ethanol, glycerol and oleic acid (Figure 5.10B). In contrast, *Cgga1Δ* mutant, disrupted for a component of GPCR-mediated signaling, was proficient in utilizing non-fermentable carbon sources (Figure 5.10B). Notably, growth defects of *Cgups15Δ* and *Cgups34Δ* mutants on non-
fermentable carbon sources were rescued upon complementation with respective ORFs (Figure 5.10B). These data indicate an important role for PI-3 kinase-mediated signaling in utilization of alternate carbon sources. Presumably, intracellular macrophage environment is nutrient-poor wherein fatty acids act as principal energy reservoir for intracellular pathogens (Lorenz and Fink, 2002). It is likely that PI-3 kinase-mediated signaling assists C. glabrata cells utilize non-fermentable carbon sources for their survival in intracellular macrophage environment.

5.2.6 Cgyps15Δ and Cgyps34Δ mutants display enhanced secretion of carboxypeptidase Y and Epa1

In S. cerevisiae, PI-3 kinase regulates protein sorting events in endocytic and secretory pathways (Bryant et al., 1998; Stack et al., 1995b). To investigate if Cgyps15Δ and Cgyps34Δ mutants are defective in protein sorting events, we selected two candidate proteins, Epa1 and carboxypeptidase Y, and monitored their trafficking to cell wall and vacuoles, respectively. Epa1 is a member of cell surface-associated adhesin protein family in C. glabrata (Cormack et al., 1999). Epa1 is a glucan-cross-linked cell-wall protein which binds to asialo-lactosyl-containing carbohydrates present on host cell surface (Cormack et al., 1999). In C. glabrata, Epa1 on the cell surface are processed by aspartyl proteases, removed from the cell wall and released to the medium (Kaur et al., 2007). Using the Cgyps15Δ and Cgyps34Δ mutants generated in this study, my colleague, Sriram Balusu, demonstrated elevated expression of Epa1 on cell surface of Cgyps15Δ and Cgyps34Δ mutants.

Further, we examined the processing and sorting of carboxypeptidase Y in wild-type and PI-3 kinase mutants. In S. cerevisiae, carboxypeptidase Y is synthesized as an inactive precursor of ~82 kDa which undergoes sequential processing to form a mature 61 kDa enzyme (Valls et al., 1987). Final maturation of pro-carboxypeptidase Y occurs in vacuolar proteinases A (Pep4) and B (Prb1)-dependent manner in the vacuole (Valls et al., 1987). Defective protein sorting processes result in mislocalization and altered processing of carboxypeptidase Y (Li and Kane, 2009; Rothman and Stevens, 1986). In C. glabrata, carboxypeptidase Y is encoded by the ORF CAGL0M13651g. First, we tested the level of carboxypeptidase Y (~59 kDa) in Cgyps15Δ, Cgyps34Δ and Cgpa1Δ mutants in log and stationary phase-grown cells. As shown in Figure 5.11A, stationary phase cells displayed
elevated levels of carboxypeptidase Y in all strains. However, total amounts of CgCPY were lower in Cgyps15Δ and Cgyps34Δ mutants compared to those in wild-type cells under both log and stationary phase growth conditions (Figure 5.11A). In contrast, no significant change in levels of carboxypeptidase Y was observed in Cggspa1Δ cells. Notably, carboxypeptidase Y in PI-3 kinase mutants displayed slightly higher molecular weight (~ 61 kDa) indicating improper processing of carboxypeptidase Y (Figure 5.11A).

Intracellular macrophage environment poses a challenge to internalized C. glabrata cells by exposing them to adverse conditions including limited nutrient availability, acidic pH and generation of reactive oxygen species. Hence, it is plausible that C. glabrata cells may require differential sorting of proteins involved in stress tolerance when engulfed by macrophages. Carboxypeptidase Y is used as model protein to study protein sorting in eukaryotes (Bryant and Stevens, 1998). To investigate if protein sorting is altered in C. glabrata cells upon macrophage internalization, we measured levels of carboxypeptidase Y in 2 and 6 h RPMI-grown and macrophage-coincubated C. glabrata cells. Levels of carboxypeptidase Y were found to be ~ 2-fold higher in macrophage-internalized cells post 2 and 6 h coincubation compared to RPMI-grown cells suggesting either increased CPY production or altered CPY sorting upon macrophage internalization (Figure 5.11B).

Figure 5.11. PI-3 kinase defective C. glabrata mutants display higher levels of vacuolar hydrolase CgCpy. (A) Protein extracts of desired C. glabrata strains, grown till indicated time points in YNB medium, were resolved on 12% SDS-PAGE gel and probed
with anti-Cpy and anti-Gapdh antibodies. Asterisk mark indicates a shift in the molecular weight of Carboxypeptidase Y. **(B)** Wild-type *C. glabrata* cells were harvested from RPMI medium and THP-1 macrophages at indicated time points and protein extracts were prepared. Protein extracts were resolved on 12% SDS-PAGE gel and probed with anti-Cpy and anti-Gapdh antibodies.

Further, to test secretion defects in PI-3 kinase mutants, we examined the secretion of carboxypeptidase Y and Epa1 in wild-type, *Cgvps15Δ*, *Cgvps34Δ* and *Cggpa1Δ* mutants by colony blot analysis. Notably, colony blot assay detects only secreted proteins and is unable to measure cytosolic proteins. As shown in Figure 5.12, colony blot analysis indicated that carboxypeptidase Y and Epa1 secretion was ~2- to 3-fold higher in *Cgvps15Δ* and *Cgvps34Δ* cells compared to wild-type cells. In contrast, no significant differences in levels of secreted carboxypeptidase Y and Epa1 were observed in *Cggpa1Δ* cells. Secretion of these two proteins was abolished in complemented strains (Figures 5.12A & B). Taken together, these data demonstrate that carboxypeptidase Y is missorted and secreted out in *Cgvps15Δ* and *Cgvps34Δ* mutants. Additionally, presence of higher amounts of secretory Epa1 may reflect a defect either in incorporation in the cell wall or increased Epa1 expression leading to misorting.

![Figure 5.12](image)

**Figure 5.12.** PI-3 kinase defective *C. glabrata* mutants display altered secretion of carboxypeptidase Y and Epa1. Indicated *C. glabrata* strains were grown in YPD medium and cells equivalent to 0.3 OD<sub>600</sub> were spotted on CAA plates. Nitrocellulose membranes were overlaid on the spots and plates were incubated overnight at 30°C. Colony
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blot assay was performed using anti-Cpy (A) and anti-Epa1 (B) antibodies. Control lane represents the spotting of equal number of yeast cells of each strain for colony blot assay. Since Gapdh is a cytosolic protein, it is not detected in colony blot assay. Colony blot with anti-Gapdh antibody was performed to rule out possibility of cell lysis.

5.2.7 *Cgyps15Δ* and *Cgyps34Δ* mutants are hyperadherent to Lec-2 epithelial cells

In *C. glabrata*, adhesins of Epa family mediate binding to host epithelial surfaces during infections (Cormack *et al.*, 1999). Previously, we demonstrated that *Cgyps15Δ* and *Cgyps34Δ* cells display higher levels of secreted and cell-surface associated Epa1. To examine if elevated level of Epa1 adhesin in PI-3 kinase mutants translate into increased adherence to epithelial cells, we measured the adherence of wild-type, *Cggpa1Δ*, *Cgyps15Δ* and *Cgyps34Δ* cells to Lec-2 epithelial cells. For these experiments, we used *Cgyps1-11Δ* strain as positive control since this strain is reported to be hyperadherent due to altered processing of surface-associated adhesin, Epa1 (Kaur *et al.*, 2007). Lec-2 is an epithelial cell line derived from the ovarian epithelium of the Chinese Hamster and widely used in adherence related studies (Deutscher *et al.*, 1984). As shown in Figure 5.13, wild-type *C. glabrata* cells displayed 35-40% adherence to Lec-2 epithelial cells while *Cgyps15Δ* and *Cgyps34Δ* mutants showed approximately 2-fold higher, 70% adherence (Figure 5.13). In contrast, only 40% of *Cggpa1Δ* mutant cells were found to be adherent to Lec-2 cells indicating that inhibition of GPCR-mediated signaling has no effect on expression, localization and function of Epa adhesins. Expression of *CgVPS15* and *CgVPS34* in *Cgyps15Δ* and *Cgyps34Δ* mutants, respectively, led to wild-type-like adherence to Lec-2 epithelial cells (Figure 5.13).
Figure 5.13. Cgps15Δ and Cgps34Δ mutants are hyperadherent to Lec-2 epithelial cells. Indicated C. glabrataΔ strains were grown overnight in CAA medium, labeled with S35(Met:Cys-65:25)-labeling mix and coincubated with fixed Lec-2 cells. Percentage adherence was determined by normalizing the radioactive counts of the lysates to respective inputs. Cgps1-11Δ strain was used as positive control in all adherence assays. Data presented are the average of 3-5 independent experiments and error bars represent standard error of mean.

5.2.8 PI-3 kinase is required for virulence of C. glabrata

To examine if PI-3 kinase-mediated signaling is essential for virulence of C. glabrata, we utilized a murine model of systemic candidiasis. For this, we examined the organ fungal load in BALB/c female mice after 7 days post intravenous injection with C. glabrata wild-type, Cgps15Δ, Cgps34Δ and Cggap1Δ cells. Cgps15Δ and Cgps34Δ-infected mice exhibited 10⁴-fold lower CFUs in kidney compared to wild-type-infected mice. Fungal burden in liver and spleen of Cgps15Δ and Cgps34Δ-infected mice was 10³-fold. In brains of Cgps15Δ and Cgps34Δ-infected mice, fungal burden was found to be ~10⁵-fold lower than that of the wild-type-infected mice. In contrast, we did not detect a significant difference in fungal burden in kidney, liver, spleen and brain between Cggap1Δ and wild-type-infected BALB/c mice (Figure 5.14). Expression of CgVPS15 and CgVPS34 from the plasmids led to wild-type-like organ fungal load for PI-3 kinase mutants in BALB/c mice suggesting
that clearance of PI-3 kinase mutants in mice was due to the disruption of CgVPS15 and CgVPS34 ORFs. These observations suggest that both regulatory and catalytic subunit of PI-3 kinase are essential for virulence in C. glabrata.

![Figure 5.14](image)

**Figure 5.14.** *Cgyps15Δ* and *Cgyps34Δ* mutants exhibit attenuated virulence in murine model of systemic candidiasis. BALB/c mice were infected with 4X10⁷ C. glabrata cells intravenously and sacrificed 7 days after infection. Appropriate dilutions of organ homogenates were plated on YPD medium and fungal burden in liver, kidneys, spleen and brain was determined by CFUs counting. Diamond and bar represent CFUs recovered from the target organs for individual mice and the geometric mean (n = 12–16) of the CFUs per organ, respectively.
5.3 Discussion

Screening of a *C. glabrata* Tn7 insertional mutant library for altered survival profiles in THP-1 macrophages identified 56 genes which were required for survival and/or replication of *C. glabrata* cells in macrophages (Chapter 3). Ten of the identified genes encoded proteins implicated in intracellular vesicular transport (Chapter 3). In the current study, we focused on the role of PI-3 kinase in virulence of *C. glabrata*. We report that deletion of either of the two subunits, regulatory subunit Vps15 or catalytic subunit Vps34, makes *C. glabrata* susceptible to host macrophage antimicrobial response. We show for the first time that mutants disrupted for PI-3 kinase colocalize with acidified lysosomes indicating their inability to modulate phagolysosomal maturation upon internalization by THP-1 macrophages. Not surprisingly, our data demonstrated that Cgyps15Δ and Cgyps34Δ mutants were unable to survive in THP-1 and primary murine macrophages. We also showed that PI-3 kinase is required for sorting of proteins, carboxypeptidase Y and Epa1, and virulence in *C. glabrata*. Notably, an essential role of PI-3 kinase in virulence of *C. glabrata* is consistent with earlier findings. In *C. neoformans*, vps34Δ mutant was defective in PI-3 kinase-mediated autophagy and exhibited attenuated virulence in murine model of infection (Hu *et al.*, 2008). Consistently, deletion of VPS34 in *C. albicans* led to impaired morphological switching, hypersensitivity to osmotic and temperature stresses and loss of virulence in murine model of systemic candidiasis (Bruckmann *et al.*, 2000).

In *S. cerevisiae*, PI-3 kinase is composed of two subunits, namely, regulatory subunit Vps15 and catalytic subunit Vps34 (Stack *et al.*, 1993). Vps15 is a serine-threonine kinase, which phosphorylates and recruits catalytic subunit Vps34 from cytoplasm to the endosome membrane. Phosphorylation of Vps34 by Vps15 is an indispensable event for its catalytic activity (Stack *et al.*, 1995a). In yeast, PI-3 kinase regulates diverse cellular functions including intracellular vesicular trafficking, autophagy and stress response (Herman *et al.*, 1991; Stack *et al.*, 1995b). Although role of regulatory subunit of PI-3 kinase, Vps15, is well-studied in the nonpathogenic yeast *S. cerevisiae*, its role in pathogenic fungi remains to be investigated.

Inhibition of phagolysosomal maturation is one of the key strategies used by pathogens to avoid phagocytes-mediated killing (Walburger *et al.*, 2004). To
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counteract host macrophage response, *C. glabrata* cells have been reported to prevent acidification of phagosomes (Seider *et al.*, 2011). Compromised ability of *Cgvsps15Δ* and *Cgvsps34Δ* mutants to prevent phagolysosomal maturation suggests that yeast PI-3 kinase contributes to modulation of acidification of *C. glabrata* containing phagosomes. Phospholipid metabolism plays a pivotal role in phagocytic cells to trigger phagocytosis (Minakami *et al.*, 2010). Modulating the host PI-3 kinase signaling may help pathogenic organism arrest phagolysosomal maturation in phagocytic cells (Vergne *et al.*, 2004). For example, *Mycobacterium tuberculosis* produces phosphatidylinositol analog phosphatidylinositol mannoside to arrest phagosomal maturation (Vergne *et al.*, 2004). Similarly in *Caenorhabditis elegans*, apoptotic cells are degraded during post zygotic development wherein apoptotic cells are engulfed by phagocytes and degraded in phagosomes (Lu *et al.*, 2011). Increased synthesis of phosphatidylinositol-3-phosphate is a prerequisite for the phagosome-mediated degradation of apoptotic cells in *C. elegans* (Lu *et al.*, 2011). It is plausible that factors required to modulate phagolysosomal acidification in *C. glabrata* are either targeted to cell surface or secreted via PI-3 kinase signaling. Deletion of PI-3 kinase may result in improper sorting of these molecules, thereby, affecting the ability of *C. glabrata* cells to modulate phagosome acidification.

A fully functional Vps15p-Vps34p complex is a prerequisite for the efficient delivery of proteins to the vacuole (Stack *et al.*, 1995b). Regulatory role of Vps15 on PI-3 kinase has not been studied in any fungal pathogen. In this study, we investigated role of *CgVPS15* in pathophysiology of *C. glabrata*. Our data suggested that both *Cgvsps15Δ* and *Cgvsps34Δ* secreted high amounts of cell surface-associated CgEpa1 and vacuolar-resident carboxypeptidase Y suggesting protein sorting defects in PI-3 kinase mutants.

Notably, macrophage-internalized *C. glabrata* cells displayed elevated levels of carboxypeptidase Y. *C. albicans* display an amino acid deprivation response upon neutrophil internalization (Ifat Rubin-Bejerano *et al.*, 2003). Similarly, amino acid biosynthetic genes were found to be up-regulated in macrophage-internalized *C. glabrata* cells (Chapter 4). It is plausible that elevated carboxypeptidase Y contribute to buffer the amino acid pool in nutrient-deprived macrophage environment. In accord, we observed higher carboxypeptidase Y in stationary-phase grown *C. glabrata* cells. Based on above findings, we hypothesize that active PI-3 kinase
complex might be essential for proper trafficking of molecular effectors required to survive host antimicrobial response. Notably, \( \text{Cggpa1}\Delta \) cells did not show any defect in proliferation in THP-1 macrophages and murine model of systemic candidiasis suggesting that GPCR signaling may not be essential for pathogenesis of \( C. \text{glabrata} \).

In yeast, Vps34 constitutes two PI-3 kinase complexes (Kihara et al., 2001). Atg14 and Vps38, which represent unique components of these complexes, regulate autophagy and carboxypeptidase-Y processing, respectively. Importantly, autophagy and pexophagy are implicated in mobilization of intracellular resources, a critical virulence determinant, in survival of \( C. \text{glabrata} \) in murine macrophages (Roetzer et al., 2010). However, autophagy defect has been shown to cause only 40-50% diminished proliferation in macrophage model (Roetzer et al., 2010) suggesting the existence of additional cellular mechanisms required for survival and/or proliferation of \( C. \text{glabrata} \) in macrophages. Although \( C. \text{glabrata} \) possesses the orthologs of \( S. \text{cerevisiae} \) Atg14 and Vps38 proteins (http://genolevures.org/cagl.html#), it remains to be determined whether roles for the two PI-3 kinase complexes are conserved between \( S. \text{cerevisiae} \) and \( C. \text{glabrata} \).

In conclusion, our data establish PI3-kinase as a pivotal virulence determinant of \( C. \text{glabrata} \).