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Saccharomyces cerevisiae Gpi2, an accessory subunit of the enzyme catalyzing the first step of glycosylphosphatidylinositol (GPI) anchor biosynthesis, selectively complements some of the functions of its homolog in *Candida albicans*

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Abstract *GPI2* encodes for one of the six accessory subunits of the GPI-*N*-acetylglucosaminyltransferase (GPI-GnT) complex that catalyzes the first step of GPI biosynthesis in *S. cerevisiae* and *C. albicans*. It has been previously reported in *S. cerevisiae* that this subunit physically interacts with and negatively modulates Ras signaling. On the other hand, studies from our lab have shown that the homologous subunit in *C. albicans* is a positive modulator of Ras signaling. Are the functions of this subunit therefore strictly species dependent? We present here functional complementation studies on *GPI2* from *S. cerevisiae* and *C. albicans* that were carried out to address this issue. Expression of *CaGPI2* in a *ScGPI2* conditional lethal mutant could not restore its growth defects. Likewise, *ScGPI2* overexpression in a *CaGPI2* heterozygous mutant could not restore its deficient GPI-GnT activity or reverse defects in its cell wall integrity and could only poorly restore filamentation. However, interestingly, *ScGPI2* could restore lanosterol demethylase (*CaERG11*) levels and reverse azole resistance of the *CaGPI2* heterozygote. It appeared to do this by regulating levels of another GPI-GnT subunit, *CaGPI19*, which we have previously shown to be involved in cross-talk with *CaERG11*. Thus, the effect of *CaGPI2* on sterol biosynthesis in *C. albicans* is independent of its interaction with the GPI-GnT complex and Ras signaling pathways. In addition, the interaction of Gpi2 with other subunits of the GPI-GnT complex as well as with Ras signaling appears to have evolved differently in the two organisms.

Keywords Glycosylphosphatidylinositol · GPI-*N*-acetylglucosaminyltransferase complex · *C.albicans* · *S.cerevisiae* · Gpi2 · Functional complementation · Morphogenesis · Ergosterol biosynthesis

Introduction

Glycosylphosphatidylinositol (GPI) anchors are integral to the eukaryotic world, being used to anchor a variety of proteins to the extracellular face of the cell membrane or the cell wall. A number of these proteins, in a pathogenic fungus like *C. albicans*, participate in communication with the environment, responding to external stimuli, establishing colonies or infection and causing virulence. The pathway appears to be essential in lower eukaryotes, affecting growth and viability. In eukaryotic pathogens in general, defects in GPI biosynthesis also significantly attenuates virulence, making them interesting targets from the clinical point of view [1].

The biosynthesis of the GPI anchor precursor and its attachment to the various proteins that carry GPI anchor attachment signals occurs in the endoplasmic reticulum. While most of the steps of the sequential GPI biosynthetic pathway have been identified and the genes/enzymes catalyzing them are known, the details of the mechanisms or how this pathway is regulated and why several steps appear to have catalytic subunits assisted by accessory ones are not very clear [2]. In a recent set of studies, we have analyzed the possible roles of

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two of the accessory subunits of the GPI-*N*-acetylglucosaminyltransferase (GPI-GnT) enzyme complex that catalyzes the first and committing step of the pathway in *C. albicans* involving transfer of *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc to phosphatidylinositol (PI) [3–5]. We have shown that each of these subunits is important for GPI biosynthesis. In addition, they have specific effects on ergosterol biosynthesis and hyphal morphogenesis in the organism. Interestingly, though, the effects of *CaGPI2* downregulation are quite contrary to that of *CaGPI19* downregulation. While the latter resulted in depletion of membrane ergosterol levels due to downregulation of lanosterol demethylase (*CaERG11*), a key enzyme in sterol biosynthesis, the former resulted in upregulation of *CaERG11* and increase in ergosterol content. Likewise, the latter caused hyperfilamentation due to Ras hyperactivation while the former caused hypofilamentation due to reduced Ras activity. We further went on to show that these opposite phenotypes were exhibited due to the fact that the two genes, *CaGPI2* and *CaGPI19*, were themselves negatively co-regulated. However, both genes did not simultaneously talk to the two pathways; while *CaGPI2* predominantly talked to Ras signaling and dictated hyphal morphogenesis, *CaGPI19* mainly affected the sterol biosynthetic pathway.

Although ours was the first study to report a cross-talk between GPI biosynthesis and sterol biosynthesis in any organism, there have been previous studies from *S. cerevisiae* that have indicated a link between GPI biosynthesis and Ras signaling. Indeed, some of the GPI-GnT subunits of the organism too have an effect on Ras signaling. Both Gpi2 and Eri1 subunits have been shown to physically interact with Ras2 and repress its activity [6]. Thus, *gpi2* and *eri1* mutants in *S. cerevisiae* were hyperfilamentous [6]. So also were the *gpi1* and *gpi19* deletion mutants [6,7], suggesting that these subunits may all have similar effects on Ras signaling. This appeared quite in contrast to what we saw in *C. albicans*, a close relative of *S. cerevisiae*.

Thus, in order to understand how these species-specific differences may be important for the two organisms, in this study we focused on understanding how well ScGpi2 would complement the functions of CaGpi2. We explored three different functional aspects of CaGpi2. We introduced *ScGPI2* in a heterozygous mutant of *CaGPI2* in *C. albicans* and first asked whether it could restore the catalytic activity of the GPI-GnT complex. We next inquired whether the *S. cerevisiae* homolog would be able to reverse the azole resistance phenotype of the *CaGPI2* heterozygote and restore its *CaERG11* transcript levels to near normal levels. And in the end we asked whether it could restore filamentation in the hypofilamentous *CaGPI2* mutant. We discovered that ScGpi2 could correctly take over the function of CaGpi2 when it came to its cross-talk with the ergosterol biosynthetic pathway or the restoration of *CaGPI19* transcription levels. However, this cross-talk did not depend on a functional GPI-GnT complex formation, since the heterozygous *CaGPI2* strain overexpressing *ScGPI2* could not recover GPI biosynthetic activity. Further, *ScGPI2* could only poorly restore Ras signaling in the *CaGPI2* heterozygote. A Clustal Omega analysis suggested that the CaGpi2 had 27 extra residues at its N-terminus as compared to ScGpi2. Hence we generated a deletion mutant of CaGpi2, ΔN-*CaGPI2*, lacking these 27 residues and also looked to see whether it would behave like the ScGpi2. We learned that ΔN-*CaGPI2* was functionally equivalent to *CaGPI2*. In other words, the functional differences between ScGpi2 and CaGpi2 were not due to the longer N-terminal domain of the *C. albicans* homolog.

Materials and methods

Materials All scientific grade chemicals were purchased from Sigma Aldrich, Qiagen, Merck or SRL. Growth media, enzymes, DNA markers and primers were purchased from Himedia, Fermentas, Bangalore Genei and Sigma respectively. UDP-[6^3H] GlcNAc was purchased from American Radiochemicals. HRP-conjugated Anti-GFP was obtained from Santacruz Biotechnology.

Strains and growth conditions: C. albicans strains used in the study have been listed in Table 1. All the strains were grown at 30 °C in YEPD or synthetic defined (SD) medium. Hyphal analysis was performed by growing the strains on Spider agar plate at 37 °C. The bacterial strain DH5α used for cloning and purification of plasmids were grown in LB medium at 37 °C.

Construction of various strains The primers used for generating the vectors and strains used in this study are given in Table 2.

Cloning and generation of *C. albicans* mutants-Δ*N-CaGPI2* (lacking 1–81 nucleotide residues) was amplified from the construct pACT1-*CaGPI2* (full *CaGPI2*) which was earlier generated in the lab [3]. Amplified Δ*N-CaGPI2* was inserted in pACT1-GFP vector between HindIII and NheI restriction. The pACT1-Δ*N-CaGPI2* construct was linearized by StuI and used to transform *CaGPI2* heterozygous mutant using lithium acetate (LiAc) method [8]. The colonies were selected on SDUra$^-$ minimal medium. The colonies were confirmed by PCR using ΔN-CaGPI2 FP and RPS10 RP. The amplification of ~1.0 kb confirmed the integration of Δ*N-CaGPI2* at *RPS10* locus. Similarly, pACT1-*ScGPI2* construct was generated. *S. cerevisiae GPI2* was amplified from genomic DNA of haploid *S. cerevisiae* YPH500 strain using ScGPI2 FP and ScGPI2 RP primers.

C-terminally tagged ScGpi2 and CaGpi2 for protein expression studies were generated using pEGFP vector (a kind gift from Prof. Alistair JP Brown, Institute of Medical

Table 1 *C. albicans* and *S. cerevisiae* strains and mutants used in this study

Strains	Reference in text	Source
CAI4	CAI4	[10]
CAI4-*Cagpi2*/*CaGPI2*	Hz	[3]
CAI4-*Cagpi2*/*CaGPI2*/P_{ACT1}-*CaGPI2*	Hz/*CaGPI2*	[3]
CAI4-*Cagpi2*/*CaGPI2*/P_{ACT1}-Δ*NCaGPI2*	Hz/Δ*N-CaGPI2*	This study
CAI4-*Cagpi2*/*CaGPI2*/P_{ACT1}-*ScGPI2*	Hz/*ScGPI2*	This study
CAI4-P_{ACT1}-GFP (Vector)	CAI4/Vector	[3]
YPH-GAL1-*ScGPI2*	YPH-GAL1-*ScGPI2*	This study
YPH-GAL1-*ScGPI2*/YEp-*CaGPI2*	YEpHIS/*CaGPI2*	This study
YPH-GAL1-*ScGPI2*/YEp-ΔN-*CaGPI2*	YEpHIS/Δ*N-CaGPI2*	This study
YPH-GAL1-*ScGPI2*/YEpHIS	YEpHIS only	This study
CAI4-*Cagpi2*/*CaGPI2*/P_{ACT1}-*CaGPI2-EGFP*	Hz/*CaGPI2-EGFP*	This study
CAI4-*Cagpi2*/*CaGPI2*/P_{ACT1}-*ScGPI2-EGFP*	Hz/*ScGPI2-EGFP*	This study

Sciences, University of Aberdeen, U.K.) in which *ADH1* promoter was replaced with *ACT1* promter. The gene was cloned along with *ACT1* promoter between restriction sites Xho1 and Pst1. The constructs were then transformed in *CaGPI2* heterozygous mutant using pACT1-Xho1 FP and CaGPI2/ScGPI (Pst1) RP (listed in the Table 2) Protein expression in the cells was monitored under a Nikon ANDOR spinning disk confocal microscope. Since *C. albicans* cells tend to show some background fluorescence in the same wavelength range as GFP emission, the heterozygous *CaGPI2* strain provided a useful control. Protein expression was also monitored using dot-blot assays. HRP-conjugated monoclonal anti-GFP was used for this and chemiluminiscence was detected using luminol and hydrogen peroxide.

Cloning and generation of *S. cerevisiae* mutants-The construct pTZ-*URA3*-*GAL1*, for PCR-mediated replacement of native promoter in *S. cerevisiae* by conditionally regulatable *GAL1* promoter, was earlier made in the lab [4].

Table 2 List of primers used in the study

Primer Name	Primer sequence
ΔN-CaGPI2 FP (HindIII)	GCGAAGCTTATGCAAGAGCAACCATCATGG
CaGPI2 RP (NheI)	GCGGCTAGCTCAGCTTTGTATACTTGACTTCAT
ScGPI2 FP (HindIII)	GCGAAGCTTATGACAAGATCTCCCTGGAAG
ScGPI2 RP (NheI)	GCGGCTAGCCTAATCCAATATTGGTGTTCTT
ΔNCaGPI2 FP (BamHI)	GCGGGATCCATGCAAGAGCAACCATCATGG
CaGPI2 RP (MluI)	GCGACGCGTTCAGCTTTGTATACTTGACTTCAT
RPS10 RP	TTTCTGGTGAATGGGTCAACGAC
GAL1-ScGPI2 FP	ATCTTGATATCCGAACTTGAGACCACTTCGCGGCTACTAAATTTAT GAACT AAAGGGTAATAACTGATATAATTAAAT
GAL1-ScGPI2 RP	TATCTGGGTACTCCTGTTTCAACCATGTAGGCGCTTCCAGGGAG ATCT TGTCATGGTTTTTTCTCCTTGACGTTAAAG
YEp-LEU FP	GGGATCCATGATATTCCATTTTAACCAA
YEp-LEU RP	ACGCGTTCATTCATATAGAACATCATTCAC
CaGPI2 RP (Pst1)	GCGCTGCAGTTGCTTTGTATACTTGACTTCATTAACTTTGGTTTCGCC
ScGPI2 RP (Pst1)	GCGCTGCAGTTATCCAATATTGGTGTTCTTGCATCCCATGT
pACT1-Xho1 FP	GCGCTCGAGCGATAGAGCTATTAAGATCACC
ScGPI2 RT FP	GTCTTTTTGGCTGACCCTATGG
ScGPI2 RT RP	TGGTGGAAAGGTTAGAGGGTTT
GAPDH RT FP	CAGCTATCAAGAAAGCTTCTG
GAPDH RT RP	GATGAGTAGCTTGAACCCAA
ERG11 RT FP	GGAACGAGATTGTAAGATAG
ERG11 RT RP	AGTACATTGGGTCATTTCAG
GPI2 RT FP	GGCCAATAGCATTTCTAC
GPI2 RT RP	CCATCACAAAAACAGACAAA

The *URA3-GAL1* fragment was amplified by GAL1-*ScGPI2* FP and GAL1-*ScGPI2* RP having 50 bp overhangs homologous to −250 to −200 upstream of *ScGPI2* and +1 to +50 from start of gene, respectively. The PCR amplified product was used to transform the haploid YPH500 strain of *S. cerevisiae* and plated on either SDUra$^-$Galactose (4 %) or SDUra$^-$ Glucose (2 %). The colonies obtained after transformation were initially screened by comparing their growth in permissive (2 % galactose) and repressive (2 % glucose) growth medium. The colonies showing growth defects in repressive growth medium were further confirmed by PCR using GAL1-ScGPI2 FP and GAL1-ScGPI2 RP.

Complementation studies in YPH-GAL1-ScGPI2: For complementation analysis of *CaGPI2* in *S. cerevisiae*, two constructs were made, *YEp-CaGPI2* and *YEp-ΔN-CaGPI2*. *ΔN-CaGPI2* was amplified by using ΔN-CaGPI2 FP and CaGPI2 RP and inserted in the yeast expression vector, YEpHIS [9], between BamHI and MluI restriction enzyme sites. The *YEp-CaGPI2* construct was also similarly constructed. The purified constructs were used to transform YPH-GAL1-ScGPI2 strain using LiAc method [8] and selected on Ura$^-$Leu$^-$ minimal media with 4 % galactose and 2 % sucrose. Colonies obtained after transformation were confirmed by PCR using gene specific primers. As a control, YPH-GAL1-ScGPI2 was also transformed with the empty vector, YEpHIS. Colonies transformed using YEpHIS vector were confirmed by YEp-LEU FP and YEp-LEU RP primers. Comparisons of growth between different strains were performed to find out whether the inserted gene could rescue the growth defect in repressive conditions.

Plate assays Plate assays were performed to analyze the growth pattern and response of mutants to the presence of varied types of stress inducers within the growth medium, as described previously [4]. The wild type strain CAI4 was used as control in all these studies. This strain is auxotrophic for uridine and has a single nutritional marker *URA3* for genetic manipulation and selection [10]. And since there are reports that the *URA3* gene can influence hyphal growth and virulence [11], we have used an additional control strain in which one copy of *URA3* has been introduced at the *RPS10* locus (CAI4/Vector) for studies which involved strains that were generated using *URA3* as selection marker.

Preparation of microsomes from C. albicans and GPI-GnT activity assay Microsomes from different *C. albicans* strains were prepared as described in the previous study [3]. The quantification of protein concentration in microsomes was done using BCA Kit (Sigma Aldrich). GPI-GnT assay was done by using 1200 μg of total protein in the microsomes. UDP-[6^3H] GlcNAc in the presence of tunicamycin was provided as donor in these assays. The total glycolipids were extracted from these reaction mixtures and run on a HPTLC plates in a CH_3OH:$CHCl_3$:H_2O::25:65:4 solvent system. The total radiolabelled [6^3H] GlcNAc-PI and [6^3H] GlcN-PI were quantified using the WinScan software in a BioscanAR200 TLC scanner. Relative activity was calculated assuming that the activity in the control sample was 100 %.

Transcript level analysis using RT-PCR Transcript level analysis for the different genes was done as reported previously [12]. Briefly, 10 ml of secondary cultures which were grown for 8 h at 30 °C, 220 rpm were pelleted down and washed twice with diethylpyrocarbonate (DEPC) treated water. Trizol reagent (Sigma Aldrich) was used to extract the total RNA and 2 μg of total RNA was used to prepare cDNA. SYBR Green PCR Master Mix (Applied Biosystems) was used to quantify the transcript levels. *GAPDH* levels were taken as control in all transcript level analysis.

Morphological studies Primary cultures were grown overnight in 10 ml YEPD media at 30 °C, 220 rpm. 5 ml fresh YEPD media were inoculated using 2 % inoculums from the primary culture and were grown at 30 °C, 220 rpm till they reached the mid log phase. The $O.D._{600nm}$ was monitored and the culture was diluted to $O.D._{600nm}$ of 0.2 using 0.9 % saline. 10 μl of each strain was spotted on Spider plates and incubated at 37 °C. The hyphae formation at the edges of colonies was monitored at 2× magnification under a Nikon SMZ 1500 microscope using NIS Elements software.

Results and discussion

S. cerevisiae has often been used as a model organism. Given the close relationship that it shares with *C. albicans*, it has often been assumed that the major biochemical pathways of the two organisms would be very similar. Thus, it came as quite a surprise for us when we discovered that the regulation of Ras signaling via the first step of GPI biosynthesis in *C. albicans* [3] was not quite the same as was observed in *S. cerevisiae* [6]. Since we had previously demonstrated that the *GPI2* encoded subunit of the GPI-GnT complex was involved in talking to the Ras signaling pathway, we sought to understand how the Gpi2 proteins from the two organisms compared.

Comparison of Gpi2 sequences from *S. cerevisiae* and *C. albicans*

Gpi2 protein sequences from *S. cerevisiae* and *C. albicans* were obtained from Saccharomyces Genome Database (www.yeastgenome.org) and Candida Genome Database (www.candidagenome.org). The protein sequences were aligned

Fig. 1 Homology between Gpi2 homologs of *S. cerevisiae* and *C. albicans*. Sequence homology between CaGpi2 and ScGpi2 was studied using Clustal Omega. The transmembrane regions were predicted by TMHMM software and have been highlighted in the figure, yellow for Cagpi2 and cyan for ScGpi2. Both ScGpi2 and CaGpi2 are putative multi-tansmembrane proteins. CaGpi2 sequence is longer as compared to ScGpi2 and has an additional stretch of 27 amino acids at its N-terminus

using Clustal Omega (http://www.ebi.ac.uk/tools/msa/clustalo/) and the probable transmembrane regions were predicted using TMHMM (http://www.cbs.dtu.dk/services/TMHMM/). The amino acid sequences of Gpi2 in the two organisms are of variable lengths, each possessing 6 putative transmembrane domains (Fig. 1). However, it must be pointed out that the transmembrane domains predicted by TMHMM is not conclusive and is yet to be supported experimentally. CaGpi2 showed 34 % sequence identity to ScGpi2. Several residues across the length of the proteins appear to be conserved. Some conserved residues appear to be in the putative soluble domains while others appear to be in the putative transmembrane regions. Interestingly, as compared to ScGpi2, CaGpi2 had 27 additional amino acids at its N-terminus. Are these differences significant enough to manifest in a difference in function? In order to test this, we looked to understand whether *CaGPI2* could functionally complement *ScGPI2* and vice versa.

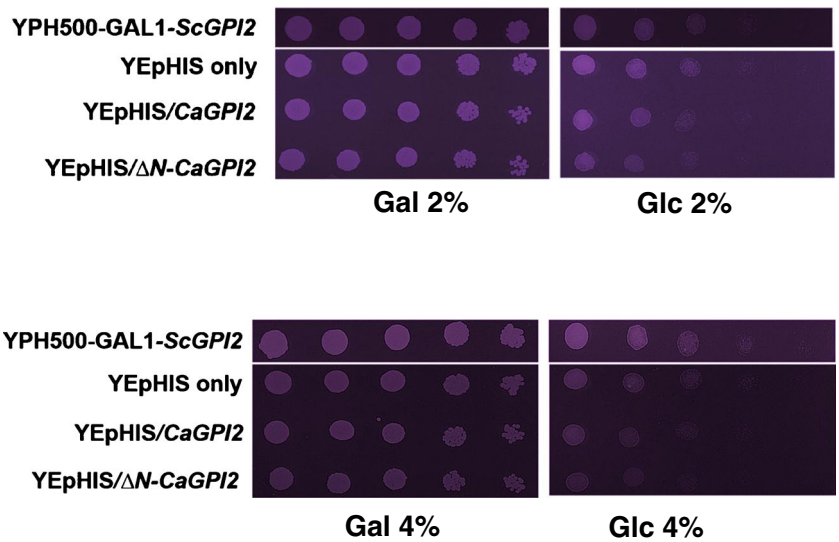

Fig. 2 Neither *CaGPI2* nor *ΔN-CaGPI2* complements a conditional null mutant of *gpi2 in S. cerevisiae* (YPH-*GAL1-ScGPI2*). Complementation study was done in haploid YPH500 strain of *S. cerevisiae* in which *ScGPI2* was placed under the regulatable *GAL1* promoter (YPH-*GAL1-ScGPI2*). For complementation studies, *CaGPI2* and Δ*N-CaGPI2* were cloned in YEpHIS vector and used to transform YPH-*GAL1-ScGPI2* strain to generate *YEpHIS*/*CaGPI2* and *YEpHIS*/Δ*N-CaGPI2* strains respectively. As a control, YPH-*GAL1-ScGPI2* was also transformed with the empty vector YEpHIS (*YEpHIS* only). Growth in permissive conditions (in presence of galactose) and in repressive conditions (in glucose), for regulating *ScGPI2* expression, is shown. YPH-*GAL1-ScGPI2* could grow well in the permissive conditions (Gal), but displayed a growth defect in the repressive conditions (Glc), thus reflecting the essentiality of *ScGPI2* for its growth. Constitutive episomal expression of *CaGPI2* or Δ*N-CaGPI2* in YPH-*GAL1-ScGPI2* mutant could not rescue its growth defect under repressive conditions, thus depicting an absence of functional complementation

In this context, it would also be relevant to point out that we had a similar situation when we had studied the *CaGPI19* gene and compared it with its homolog from *S. cerevisiae*. There too we had noticed that the *C. albicans* homolog had a remarkably longer N-terminal segment and deletion of these 151 residues had resulted in a protein that could exhibit functional complementation in *S. cerevisiae* [4].

CaGPI2 cannot complement the growth defect in a *ScGPI2* conditional lethal mutant

To check whether *CaGPI2* could complement *ScGPI2*, we first needed to make a conditional null mutant of *ScGPI2* in *S. cerevisiae*. This is because *ScGPI2* is known to be an essential gene affecting viability in *S. cerevisiae* [13]. Therefore, *ScGPI2* conditional lethal mutant was generated in *S. cerevisiae* haploid strain YPH500 (as described in Methods). The native promoter of *ScGPI2* was replaced by the conditionally regulatable *GAL1* promoter, to generate YPH-GAL1-*ScGPI2* strain. This strain exhibited growth defects in repressive conditions (in presence of glucose), when the expression of *ScGPI2* from *GAL1* promoter was inhibited (Fig. 2). Constitutive expression of *CaGPI2* or ΔN-*CaGPI2* in this strain could not rescue the growth defect. Thus, neither *CaGPI2* nor its deletion mutant could functionally complement *ScGPI2*.

Would *ScGPI2* be able to functionally complement a *CaGPI2* heterozygous mutant? This is the next question we sought to address.

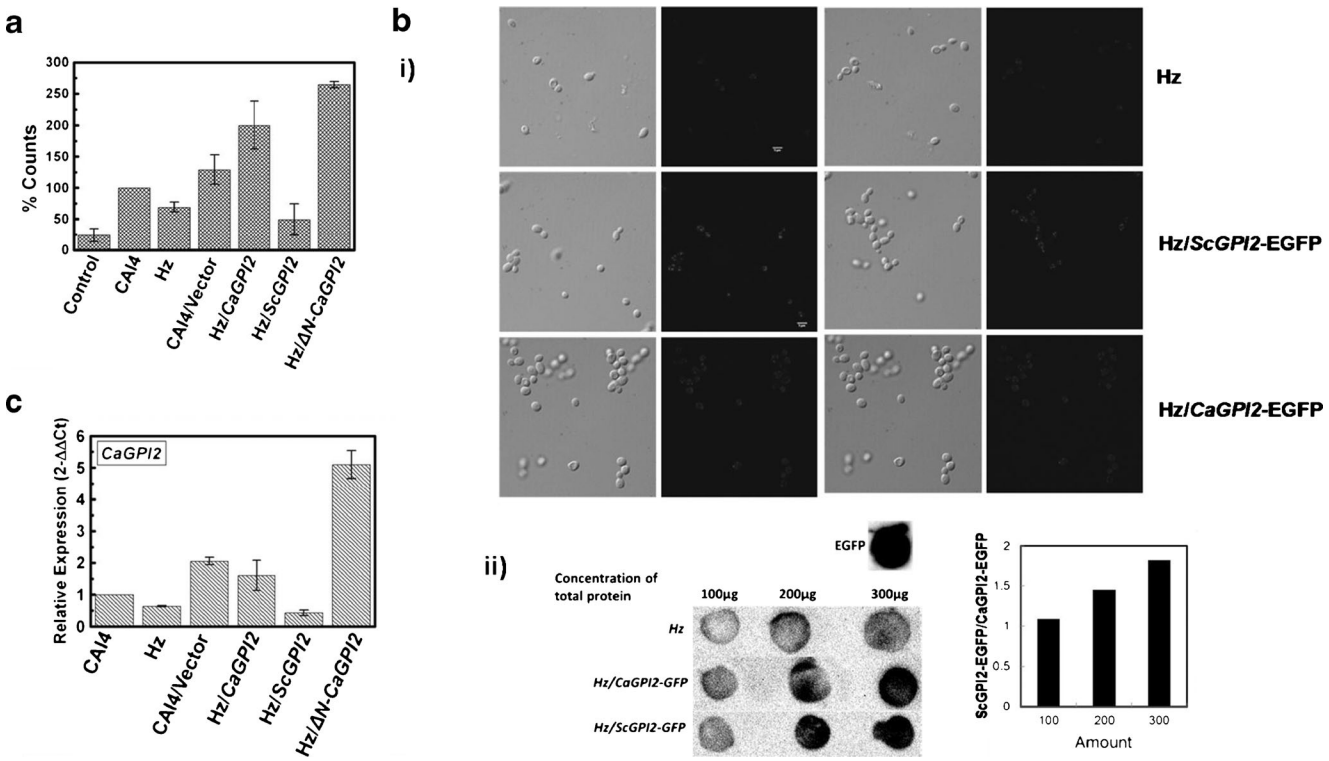

Fig. 3 GPI-GnT activity and *CaGPI2* transcript levels in *CaGPI2* heterozygous mutant are poorly complemented by *ScGPI2* but restored by Δ*N-CaGPI2*. a) GPI-GnT activity was quantified in microsomes generated from CAI4, Hz (*CaGPI2* heterozygous mutant), CAI4/Vector (CAI4 transformed with the empty vector pACT1-GFP), Hz/Δ*N-CaGPI2* (*CaGPI2* heterozygous mutant with overexpression of Δ*N-CaGPI2*) and Hz/*ScGPI2* (*CaGPI2* heterozygous mutant with overexpression of *ScGPI2*), as described in Methods section. The percentage of GPI-GnT activity in microsomes from the different strains relative to the wild type strain is shown. CAI4/Vector exhibited GPI-GnT activity comparable to CAI4 [3], whereas, Hz/Δ*N-CaGPI2* showed a higher GPI-GnT activity. There was almost no activity in Hz/*ScGPI2*. The experiment was done twice in duplicates with different microsomal preparations for confirmation. The results shown are an average of data from a single experiment done in duplicates, along with the standard deviations. b) Expression of ScGpi2-EGFP and CaGpi2-EGFP in the *CaGPI2* heterozygous mutant transformed either with *pACT1-ScGPI2-EGFP* or *pACT1-CaGPI2-EGFP*. i) Confocal microscopy images showing fluorescence of EGFP tagged proteins expressed in the cells of the different strains. The scale bar corresponds to 5 μm. ii) Dot blot images for assessing EGFP-tagged protein expression in the total cell lysate from the different strains as mentioned in the figure. HRP-conjugated anti-GFP antibody was used for binding the expressed EGFP. Hydrogen peroxide and luminol were used for chemiluminiscence observation. Left panel, the dot blots; Right panel, quantification of the relative intensities of ScGpi2-EGF versus Cagpi2-EGFP in the two strains plotted as a function of total amount of protein in each spot. **c)** *CaGPI2* transcript levels were found to be downregulated by approximately 0.6±0.1 fold (P value=0.004) in *CaGPI2* Hz, while in Hz/*ScGPI2*the levels were found to be even lower *i.e.* 0.4±0.09 (P value= 0.002). However, the levels were upregulated by approximately 5-fold in Hz/*ΔN-CaGPI2* (5.1±0.4; P value=0.001). CAI4/vector had 2-fold±0.1 (P value=0.001) higher transcript levels of *CaGPI2* than CAI4. The experiment was done twice in duplicates and average values with standard deviation are plotted

ScGPI2 cannot restore GPI-GnT activity in *CaGPI2* mutant

In our previous study we had reported that *CaGPI2* heterozygote mutant displayed reduced GPI-GnT activity and re-introduction of *CaGPI2* restored the GPI-GnT activity in the mutant [3]. We checked if the introduction of *ScGPI2* in *CaGPI2* heterozygote mutant (Hz/*ScGPI2*) could restore the defective GPI-GnT activity in the mutant. GPI-GnT activity in microsomes prepared from the different strains was assessed by providing UDP-[6^3H] GlcNAc as donor (as described in methods). We found that the *CaGPI2* heterozygous mutant continued to display reduced GPI-GnT activity even after overexpression of Sc*GPI2* (Fig. 3a). In order to confirm that this inability to complement *CaGPI2* was not due to lack of protein expression we first analyzed the transcript levels of *ScGPI2* in the cells. Using *C. albicans GAPDH* as standard, we found that using *ScGPI2* levels were approximately a 100-fold higher when expressed using the *ACT1* promoter. In comparison, *CaGPI2* transcripts were only about 80 % of the *GAPDH* level in cells expressing CaGpi2 using the same promoter. We also examined protein expression in the cells using *ScGPI2-GFP* or *CaGPI2-GFP* for complementation. As can be seen in Fig. 3b (i), when compared to the *CaGPI2* heterozygous mutant itself, or the heterozygous *CaGPI2* mutant cells expressing CaGpi2-GFP, cells expressing ScGpi2-GFP show significant levels of GFP fluorescence. The intensity of fluorescence in the cells was quantified using TiE-NIS analysis software and averaged over 40 cells. We observed that cells from the strain expressing Sc-Gpi2-EGFP had higher average fluorescence (5.8±1.1 arbitrary units (a. u.) per cell per unit area) as compared to those expressing CaGpi2-EGFP (3.9±1.2 a. u.). But the fluorescence intensity of the latter was barely above the background levels observed for the heterozygous *CaGPI2* mutant (2.6±0.8 a. u.). It must be also pointed out that the expression of *C. albicans* GPI-GnT subunits has generally been found to be low, often requiring a secondary antibody conjugated to TRITC or other fluorophores with high quantum yields for detection (unpublished data from the lab). In addition, we routinely obtained considerably higher GPI-GnT activity in microsomes made from *S. cerevisiae* as compared to those from *C. albicans* for the same amount of total protein. As can also be seen from Fig. 3b (ii), dot blot analysis confirmed that ScGpi2-GFP was expressed at levels at least comparable, if not higher than that of CaGpi2-GFP. Thus, we concluded that despite protein expression *ScGPI2* could not rescue GPI biosynthesis in the *CaGPI2* heterozygote mutant.

We also analyzed how removal of the additional 27 amino acids at the N-terminus of CaGpi2 (ΔN-*CaGPI2*) affected its role in GPI-GnT activity. To our surprise, we found that introduction of ΔN-*CaGPI2* in *CaGPI2* heterozygote mutant (Hz/ΔN-*CaGPI2*) resulted in greatly enhanced GPI-GnT activity (Fig. 3a), significantly higher than that of the wild type strain and the *CaGPI2* heterozygote in which a second copy of full length *CaGPI2*was re-introduced (Fig. 3a). In other words, at the level of GPI biosynthesis ΔN-*CaGPI2* was certainly capable of mirroring the functions of the full-length gene and also improving upon it.

What explains the higher GPI-GnT activity in the strain overexpressing ΔN-*CaGPI2*? One possible explanation would be that the N-terminal domain of the protein is actually a negative modulator of the activity of the enzyme complex and its deletion improves functionality. Alternatively, it is possible that the N-terminal segment contributes to reduced stability of the mRNA transcripts; deletion of this segment, in such a case, should result in higher transcript levels for the gene, resulting in higher levels of the protein product. We favor this latter explanation since we observed much higher transcript levels for *CaGPI2* in the strain overexpressing ΔN-*CaGPI2*as compared to that which overexpressed *CaGPI2* (Fig. 3b). Also, this appears to correlate well with all the subsequent data as well.

ScGPI2 is unable to restore cell wall integrity in *CaGPI2* heterozygote mutant

In a previous study we had also observed that the *CaGPI2* heterozygous mutant was sensitive to cell wall perturbing agents, such as CFW, Congo red and SDS [3]. We had proposed that the disruption of GPI-GnT activity led to altered cell wall, as GPI-anchored proteins form a significant fraction of cell wall proteins in *C. albicans* [14]. So here we analyzed if the introduction of *ScGPI2* in *CaGPI2* heterozygote mutant could lead to restoration of the cell wall integrity. We found

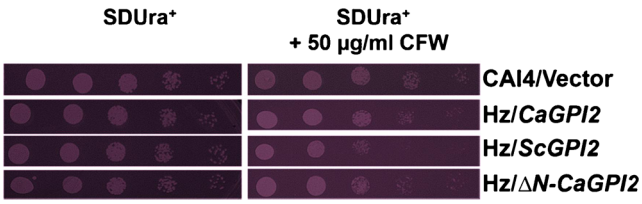

Fig. 4 Cell wall integrity in *CaGPI2* heterozygous mutant is not rescued by *ScGPI2*. Growth of the mutants in the presence of cell wall perturbing agent CFW was monitored to assess cell wall integrity. It has been previously shown that Hz (*CaGPI2* heterozygous mutant) is extremely sensitive to CFW, when compared to the wild type strain [3]. Hz/*ScGPI2* (*CaGPI2* heterozygous mutant with overexpression of *ScGPI2*) was sensitive to CFW while Hz/*CaGPI2CaGPI2* heterozygous mutant with overexpression of *CaGPI2*) and Hz/*ΔN-CaGPI2* (*CaGPI2* heterozygous mutant with overexpression of Δ*N-CaGPI2*) behaved similar to wild type control, CAI4/Vector (CAI4 transformed with the empty vector pACT1-GFP). 5 µl each of 5-fold serial dilutions starting from 0.1 $O.D._{600nm}$cells were spotted on SD-minimal CFW plates and incubated at 30 °C. The image was captured after 48 h

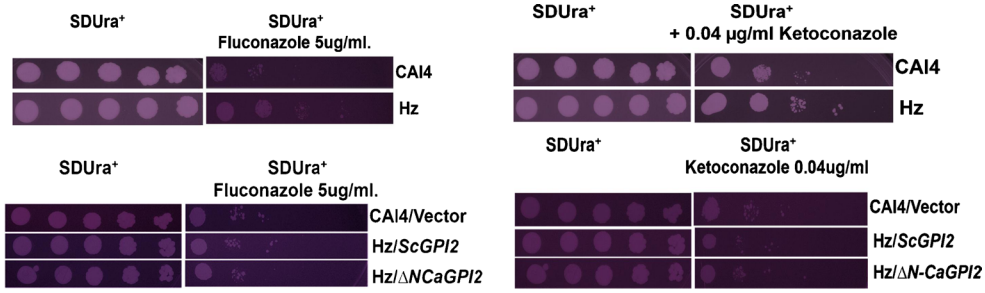

Fig. 5 Azole resistance in *CaGPI2* heterozygous mutant is reversed by *ScGPI2*. Hz (*CaGPI2* heterozygous mutant) shows resistance to azoles, when compared to the wild type strain CAI4, as also shown previously [3]. Hz/Δ*N-CaGPI2* (*CaGPI2* heterozygous mutant with overexpression of Δ*N-CaGPI2*) exhibits higher azole sensitivity as compared to CAI4/-Vector (CAI4 transformed with the empty vector pACT1-GFP), thus reversing the azole resistance observed in Hz (*CaGPI2* heterozygous mutant). Hz/*ScGPI2* (*CaGPI2* heterozygous mutant with overexpression of *ScGPI2*) also reversed the growth resistance of Hz and displayed growth similar to CAI4/Vector. 5 µl each of 5-fold serial dilutions starting from 0.1 $O.D._{600nm}$ cells were spotted on SD minimal plates with either 5 µg/ml fluconazole or 0.04 µg/ml ketoconazole and incubated at 30 °C. The image was captured after 21 h

that overexpression of *ScGPI2* in *CaGPI2* heterozygote mutant did not lead to reversal of cell wall defects and the mutant was still sensitive to cell wall perturbing agents like calcofluor white (Fig. 4). This also correlated with the inability of *ScGPI2* to restore GPI-GnT activity in *CaGPI2* heterozygote mutant.

In accordance with the restoration of GPI-GnT activity in *CaGPI2* heterozygous mutant expressing ΔN-*CaGPI2*, we found that the mutant no longer displayed sensitivity to cell wall perturbing agents (Fig. 4). Thus, restoration of GPI-GnT activity in *CaGPI2* heterozygote mutant after introduction of ΔN-*CaGPI2*, also leads to the restoration of cell wall integrity, as it does upon introduction of full length *CaGPI2*, perhaps due to the restoration of GPI anchor biosynthesis.

Expression of *ScGPI2* in C*aGPI2* heterozygote mutant can reverse the azole resistance

The*CaGPI2* heterozygote mutant also displayed azole resistance due to upregulation of *CaERG11* transcript levels, as we showed in our previous study [3]. Azole resistance and the higher *CaERG11* levels were reverted upon re-introduction of full length *CaGPI2* in the mutant. We analyzed if *ScGPI2* could also restore these phenotypes in the *CaGPI2* heterozygote mutant. We found that introduction of *ScGPI2* could indeed reverse the azole resistance in a manner comparable to the introduction of the full length *CaGPI2* in the mutant (Fig. 5). Similarly introduction of ΔN-*CaGPI2*in the heterozygous *CaGPI2* mutant resulted in a reversion of the azole resistance phenotype (Fig. 5).

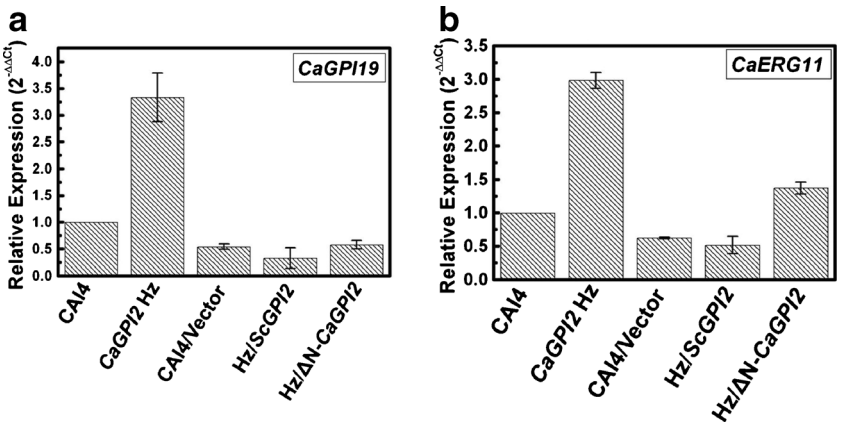

Fig. 6 *ScGPI2* can restore*CaGPI19*and *CaERG11*transcript levels in *CaGPI2* heterozygous mutant. a) The transcript level of *CaGPI19* were up regulated in Hz (*CaGPI2* heterozygous mutant) by 3.3±0.4 fold (P value=0.01), as also reported previously [3]. *CaGPI19* transcript levels were brought down to 0.3±0.2 fold (P value=0.007) fold in Hz/*ScGPI2* (*CaGPI2* heterozygous mutant with overexpression of *ScGPI2*) and 0.58 ±0.07 fold (P value=0.0001) in Hz/Δ*N-CaGPI2* (*CaGPI2* heterozygous mutant with overexpression of Δ*N-CaGPI2*), as compared to CAI4. **b)** The transcript level of *CaERG11* were found to be upregulated in Hz by 3 fold±0.1 (P value=0.03) when compared to CAI4, as also reported previously [3]. However, in Hz/*ScGPI2*, *CaERG11* transcript levels were comparable to CAI4 and CAI4/Vector (P value=0.09).Similarly, in Hz/Δ*N-CaGPI2* the transcript levels were found to be comparable to CAI4 (1.3±0.08 fold; P value=0.02). Thus *ScGPI2* and Δ*N-CaGPI2*can restore *CaERG11* and *CaGPI19* transcript levels in *CaGPI2* heterozygous mutant. The experiment was done twice in duplicates and average values with standard deviation are plotted

CaERG11 and *CaGPI19* transcript levels are restored in *CaGPI2* mutant upon introduction of *ScGPI2*

We had previously observed a negative co-regulation between *CaGPI2* and *CaGPI19* levels [3]. We had observed that *CaGPI2* heterozygote displayed enhanced *CaGPI19* transcript levels. Downregulating *CaGPI19* transcript levels led to reversal of azole resistance and also brought down the levels of *CaERG11* transcripts in the mutant [3]. We had thus concluded that the *CaGPI2* heterozygote mutant displayed azole resistance due to upregulation of *CaGPI19* transcript levels which resulted in enhanced *CaERG11* levels. Hence, we next analyzed how the introduction of *ScGPI2* in *CaGPI2* heterozygote mutant affected the levels of *CaGPI19* and *CaERG11* in the mutant.

We found that expression of *ScGPI2* in *CaGPI2* heterozygote brought back *CaGPI19* (Fig. 6a) as well as *CaERG11* transcript levels (Fig. 6b) to levels that were comparable to what was seen in the wild type strain. This also correlated well with the observed reversal of azole resistance in the *CaGPI2* heterozygote upon introduction of *ScGPI2*. In other words, when it comes to transcriptional regulation and cross-talk with the sterol biosynthetic pathway, *ScGPI2* appeared to be capable of functionally replacing *CaGPI2*.

Similarly, upon introduction of ΔN-*CaGPI2* in *CaGPI2* heterozygote, the *CaGPI19* transcript levels were reduced to roughly similar levels as the wild type strain (Fig. 6a) while the *CaERG11* levels were found to be comparable to that of CAI4 (about 1.3 fold) (Fig. 6b). Thus, ΔN-*CaGPI2* still exhibits the negative co-regulation between *CaGPI19* and *CaERG11*. Thus, the N-terminal cytoplasmic domain of CaGpi2 does not appear to be critical for the cross-talk of the first step of GPI biosynthesis with the ergosterol biosynthetic pathway.

ScGPI2 can partially restore the filamentation defect in *CaGPI2* heterozygote

A third and major role for CaGpi2 appeared to be at the level of cross-talk with Ras signaling pathway and control of filamentation, as we reported in our previous study [3]. We had observed that *CaGPI2* heterozygous mutant was hypofilamentous due to reduced Ras1-mediated signaling. As with strains exhibiting reduced Ras1-activity [15], the *CaGPI2* heterozygote was also resistant to heat shock [3]. Thus, we next explored if the expression of *ScGPI2* in *CaGPI2* heterozygote could restore the heat resistance and filamentation defect in the mutant.

We found that the heat shock resistance remained upon expression of *ScGPI2* in the *CaGPI2* mutant, suggesting incomplete restoration of Ras1-activity in the mutant

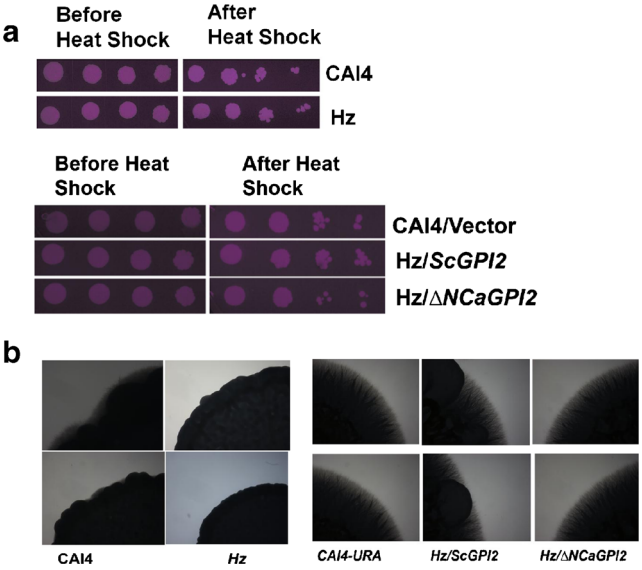

Fig. 7 Ras signaling is not rescued by *ScGPI2* but is rescued by *ΔN-CaGPI2*. a) Heat shock sensitivity was monitored to assess the Ras activity levels in the strains. We have previously shown that *CaGPI2* heterozygous mutant (Hz) shows resistance to heat shock, when compared to the wild type CAI4 strain [3]. 5 μl each of 5-fold serial dilutions of cells corresponding to 0.2 $O.D._{600nm}$was spotted before and after a heat shock of 10 min at 48 °C. Ras activity was inferred to be higher in Hz/*ΔN-CaGPI2* (*CaGPI2* heterozygous mutant with overexpression of Δ*N-CaGPI2*) as compared to wild type, CAI4/Vector (CAI4 transformed with the empty vector pACT1-GFP), since it was sensitive to heat shock. Hz/*ScGPI2* (*CaGPI2* heterozygous mutant with overexpression of *ScGPI2*), on the other hand, was resistant to heat shock suggesting that Ras activity remained low in the mutant even after introduction of *ScGPI2*. The plates were incubated at 30 °C and the image shown was captured after 56 h. **b**) *ΔN-CaGPI2* and *ScGPI2* were overexpressed in *CaGPI2* heterozygous mutant and the filamentation pattern was monitored on Spider plate at 37 °C.As a control, CAI4 was also transformed by the empty vector, pACT1-GFP (CAI4/Vector). As reported earlier [3], *CaGPI2*Hz showed lesser hyphae as compared to CAI4. Hz/*ScGPI2* was showing an unusual pattern of discontinuous hyphae surface and shorter hyphae while Hz/*ΔN-CaGPI2* showed normal hyphae as CAI4/Vector. Cells from the different strains (~1 × 10^{16}cells) were spotted on hyphae- inducing Spider medium and images were captured after 8 days

(Fig. 7a). This was accompanied by a partial reversal of the filamentation defect. The mutant overexpressing *ScGPI2* in the *CaGPI2* heterozygous background tended to show abnormal and patchy hyphal growth (Fig. 7b). These results thus suggest poor functional overlap in the regulation of morphogenesis via regulation of Ras1-signaling by Gpi2 proteins from *S. cerevisiae* as well as *C. albicans*.

Introduction of ΔN-*CaGPI2* in *CaGPI2* mutant also led to reversal of heat shock resistance (Fig. 7a) as well as complete restoration of filamentation in the mutant which was comparable to the restoration by full-length *CaGPI2* (Fig. 7b). This thus suggests that the first 27 amino acids of CaGpi2 are dispensable for its regulation of Ras1-mediated signaling as well.

Conclusions

In conclusion, it would appear that the differences between CaGpi2 and ScGpi2 are not merely due to the differences in the length of the N-terminal cytoplasmic domain. While we have not yet been able to identify where these fundamental differences may lie with respect to the amino acid sequence or the topologies of the various domains of the two proteins, we discovered that the cross-talk with the ergosterol and Ras signaling pathways were not necessarily directly dependent on an intact and functional GPI-GnT complex being formed. In effect, some of the results from our previous work too would have suggested this. Yet, this is the first time we have conclusively proven that restoration of a functional GPI-GnT complex is not necessary for the cross-talk to be established. The interaction with the Ras signaling pathway also clearly illustrates why the differences in the filamentation phenotypes of the *S. cerevisiae* and *C. albicans GPI2* mutants arise. It is clear that the heat shock resistance in the heterozygous *CaGPI2* mutant is poorly reversed by overexpression of *ScGPI2*, suggesting poor restoration of Ras signaling. Consequently, the restoration of filamentation too remains aberrant. Yet, interestingly, when it came to restoration of transcriptional regulation of *CaGPI19* and *CaERG11*, the differences between the two *GPI2* homologs appear to be rather insignificant.

How do the Gpi2 homologs from the two organisms regulate *CaGPI19* and *CaERG11* levels? One probable hypothesis would be that Gpi2 physically interacts with CaErg11 and controls its regulation in response to specific cues. That Erg11 could be physically associated with Gpi2 in *S. cerevisiae* has been suggested previously from high-throughput two-hybrid screens [16]. Our experiments with *C. albicans* had suggested that *CaERG11* is co-regulated with *CaGPI19*. Additionally, *CaGPI19* levels themselves seem to be negatively co-regulated with *CaGPI2*. In double deletion mutants, where *CaGPI2* had already been depleted, a further deletion of one copy of *CaGPI19* could continue to influence *CaERG11* levels in *C. albicans*. However, in mutants where *CaGPI19* is downregulated, a downregulation of *CaGPI2* is unable to restore *CaERG11* levels, suggesting that CaGpi19, rather than CaGpi2 is the major partner in the cross-talk with CaErg11 [3]. An alternative hypothesis would be that different functional forms of CaGpi2 are present in the cell. Thus, there could be an ER membrane localized form, which could participate in GPI-GnT complex activity, as well as physically associate with and regulate Ras-mediated morphogenesis. A different variant of CaGpi2 could also possibly be involved in interactions with transcription factors or activators/suppressors that enter the nucleus in response to specific cues (such as sterol depletion) and thus also contribute to the differential regulations of gene expression, particularly for *CaGPI19* and *CaERG11*. ScGpi2 perhaps is able to complement the functionality of this second variant of CaGpi2 but not of the first one. Whether CaGpi2 or its variant could itself relocate to the nucleus or whether it interacts with one or more factors that regulate *CaERG11* levels is currently under investigation. Identification of these probable different isoforms of Gpi2 and exploration of similar functions in other organisms could help us better establish the newly discovered additional functions of Gpi2.

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